

FORM PTO-1390 (REV 11-2000)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER CIIT1490-3	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				U.S. APPLICATION NO. (If known, specify CFR 1.5) 10/031532	
INTERNATIONAL APPLICATION NO. PCT/US00/19821		INTERNATIONAL FILING DATE 20 July 2000		PRIORITY DATE CLAIMED 19 July 1999	
TITLE OF INVENTION Detection of Biomolecules by Sensitizer-Linked Substrates					
APPLICANT(S) FOR DO/EO/US Harry B. Gray; Brian R. Crane; Jay R. Winkler; Ivan Julian Dmochowski; Jonathan Wilker; Alexander Robert Dunn					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
<ol style="list-style-type: none"> 1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below. 4. <input type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) <ol style="list-style-type: none"> a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input checked="" type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). <ol style="list-style-type: none"> a. <input type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). 7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) <ol style="list-style-type: none"> a. <input checked="" type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). Unexecuted (4 pages) 10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). 11. <input checked="" type="checkbox"/> A copy of the International Preliminary Examination Report (PCT/IPEA/409) (4 pages) 12. <input checked="" type="checkbox"/> A copy of the International Search Report (PCT/ISA/210). (9 pages) 					
Items 11 to 23 below concern document(s) or information included:					
<ol style="list-style-type: none"> 13. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 14. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 15. <input type="checkbox"/> A FIRST preliminary amendment. 16. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 17. <input type="checkbox"/> A substitute specification. 18. <input type="checkbox"/> A change of power of attorney and/or address letter. 19. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter 2 and 35 U.S.C. 1.821 - 1.825. 20. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 21. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 22. <input checked="" type="checkbox"/> Certificate of Mailing by Express Mail EV047293885US 23. <input checked="" type="checkbox"/> Other items or information: PCT Request (6 pages), Written Opinion (4 pages), Response to Written Opinion (8 pages), PCT Demand (5 pages) 					

531 Rec'd PCT/PTC 17 JAN 2002

U.S. APPLICATION NO. (if known, see 37 CFR 1.53) <div style="font-size: 1.5em; font-weight: bold;">10/031532</div>		INTERNATIONAL APPLICATION NO PCT/US00/19821		ATTORNEY'S DOCKET NUMBER CIT1490-3	
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24. <input checked="" type="checkbox"/> The following fees are submitted BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....\$1040.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO\$890.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$740.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4).....\$100.00 <div style="text-align: right; font-weight: bold;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div>				CALCULATIONS PTO USE ONLY	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e))				\$890.00 \$130.00	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	18 - 20 =	0	x \$18.00	\$..00	
Independent claims	- 3 =	0	x \$84.00	\$..00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ 280.00	\$	
TOTAL OF ABOVE CALCULATIONS =				\$1020.00	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$510.00	
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a. ☒ A check in the amount of \$510.00 to cover the above fees is enclosed.

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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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DETECTION OF BIOMOLECULES BY SENSITIZER-LINKED SUBSTRATES

5 FIELD OF INVENTION

The present invention relates to novel methods and compositions for detecting and characterizing biomolecules using sensitizer-linked substrate molecules.

10 BACKGROUND OF THE INVENTION

The ability to detect, characterize, and manipulate biomolecules in complex media is critical for understanding biochemical and metabolic processes. Methods and systems which are capable of detecting trace amounts of microorganisms, pharmaceuticals, hormones, viruses, antibodies, nucleic acids and other proteins have been based on well known binding reactions, e.g. antigen-antibody reactions, nucleic acid hybridization techniques, and protein-ligand systems. Much attention is being given to the design, synthesis, and employment of molecular probes of enzyme structure and function [Wilker, J.J. et al. *Angew. Chem. Int. Ed.* (1999), 38, 90-92; Hamachi, I. et al., *J. Am. Chem. Soc.* (1999), 121, 5500-5506; Dmochowski, I.J., et al. *Proc. Natl. Acad. Sci. USA* (1999), 96, 12987-12990; Atkinson, R.N. et al. *J. Org. Chem.* (1999), 64, 3467-3475; Tschirret-Guth, R.A. et al. *J. Am. Chem. Soc.* (1999), 121, 4731-4737; DiGleria, K. et al. *J. Am. Chem. Soc.* (1998), 120, 46-52; Murthy, Y.; Massey, V. *Meth. Enzymol.* (1997), 280, 436-460; Newcomb, M. et al. *J. Am. Chem. Soc.* (1995), 117, 3312-3313; Atkinson, J.K.; Ingold, K.U. *Biochemistry* (1993), 32, 9209-9214; Liu, K.E. et al., *J. Am. Chem. Soc.* (1993), 115, 939-947; Tschirret-Guth, R.A. et al. *J. Am. Chem. Soc.* (1998), 120, 7404-7410], owing in part to the abundance of naturally occurring cavity proteins [Tainer, J.A. et al. *J. Mol. Biol.* (1982), 160, 181-217; Bigler, T.L. et al., *Prot. Sci.* (1993), 2, 786-799; Badger, J. et al., *Proc. Natl. Acad. Sci. USA* (1988), 85, 3304-3308; Poulos, T.L. et al., *Biochemistry* (1986), 25, 5314-5322] and in part to the power of site-directed mutagenesis, to modify existing cavities and create new substrate binding sites

[Wilcox, S.K. et al., *Biochemistry* (1998), 37, 16853-16862; Goldsmith, J.O. et al., *Biochemistry* (1996), 35, 2421-2428; DePillis, G.D. et al., *J. Am. Chem. Soc.* (1994), 116 6981-6982; Fitzgerald, M.M. et al., *Biochemistry* (1994), 33, 3807-3818; Eriksson, A.E. et al., *Nature* (1992), 355, 371-373].

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Typically, detection of biomolecules of interest is performed by an observable tag or label attached to one or more of the binding elements (i.e. substrates) of the biomolecule and indicated by the presence or absence of the observable tag. Of particular interest are labels that can emit energy as luminescence through photochemical, chemical, and electrochemical processes.

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The detection of specific proteins through luminescence spectroscopy should be useful in a wide variety of fields. The rise of combinatorial chemistry has necessitated the development of sensitive and rapid screens for drug-target interactions. Luminescence is ideal for rapid screening because of its speed and sensitivity. Similarly, a luminescent probe for the *in vivo* detection of enzyme expression and localization is generally useful. Examples of widely used probes include small molecule detectors for mono- and divalent cations and Green Fluorescent Protein hybrid proteins.(d. Silva, A. P. et al., *Coord. Chem. Rev.* (1999) 185-186, 297-306; Tsien, R. Y. *Annu. Rev. Biochem.* (1998) 67, 509-544; Takahashi, A. et al., *Physiol. Rev.* (1999) 79, 1089-1125). The wide usage of these techniques suggests that a method of detecting the localization and concentration of a given enzyme is highly desirable. However, few techniques currently exist that take advantage of the inherent specificity of an enzyme for its substrate.

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25 In addition, molecules with photosensitizers attached to cofactors [Hamachi, I. et al., *J. Am. Chem. Soc.* (1999), 121, 5500-5506] can rapidly deliver redox equivalents to buried active sites for potential therapeutic applications.

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Particularly important target biomolecules are oxygenases (e.g. cytochrome P450) involved in drug metabolism and many disease states, including liver and kidney dysfunction, neurological disorders, and cancer. 54 human cytochrome P450 genes have

been identified. The cytochrome P450 genes are broken down into many families and subfamilies. The first isolated human P450s were 1A1, 1A2, 2A6, 2C8, 2C9, 2D6, 2E1, 3A4, 3A5, and 4A11. The 1A family, for example, is actively studied due to its role in carcinogen activation (F.P. Guengrich, "Human Cytochrome P450 Enzymes" in
5 Cytochrome P450: Structure, Mechanism, and Biochemistry, 2nd ed. Ed. Paul R. Ortiz de Montellano, Plenum Press, New York, 1995, pp.473-536.) and would be an optimal target for characterization.

Although more than 100 mammalian microsomal P450 isozymes have been identified,
10 direct information about their structures and physiological function is lacking. The best characterized of these is, cytochrome P450_{cam} (P450). Crystal structures are available for only six P450 oxygenases (Poulos, T. L., et al. (1995) in *Cytochrome P450: Structure, Mechanism, and Biochemistry, 2nd edn*, ed. Ortiz de Montellano, P. R. (Plenum Press, New York), pp. 125-150), all but one of which are water-soluble bacterial enzymes.

15 New methods for detecting mammalian P450s and characterizing their structures (Tschirret-Guth, R. A., et al. (1999) *J. Am. Chem. Soc.* **121**, 4731-4737) would facilitate rational drug design (Ortiz de Montellano, P. R. & Correia, M. A. (1995) in *Cytochrome P450: Structure, Mechanism, and Biochemistry, 2nd edn*, ed. Ortiz de Montellano, P. R.
20 (Plenum Press, New York), pp. 305-364) and the engineering of new catalysts (Joo, H., Zhanglin, L. & Arnold, F. H. (1999) *Nature* **399**, 670-673; Stevenson, J.-A., et al. (1996) *J. Am. Chem. Soc.* **118**, 12846-12847) for use in diagnosis and/or therapy of diseases.

Another luminescent ruthenium complex [Ru(phen)₂dppz]²⁺ is nearly undetectable in
25 water but moderate in non-aqueous solvents.(Chambon, J.-C. et al., *New J. Chem.* (1985) **9**, 527-529) The discovery that this and similar compounds also emit light when intercalated into doubled stranded DNA resulted in publications, both on the original dppz complexes and on related compounds.(Friedman, A. E. et al., *J. Am. Chem. Soc.* (1990) **112**, 4960-4962; Erkkila, K. E. et al., *Chem. Rev.* (1999) **99**, 2777-2795) The
30 mechanism of this surprising effect has been elucidated to large degree. Luminescence quenching in aqueous solution seems to occur through water hydrogen bonding to dppz

cofactor, and catalyses the oxidation of arginine to NO and citrulline.(Crane, B. R. et al., *Science* (1997) **278**, 425-431; Crane, B. R. et al., *Science* (1998) **279**, 2121-2126; Raman, C. S. et al., *Cell* (1998) **95**, 939-950; Fischmann, T. O. et al., *Nature Str. Biol.* (1999) **6**, 223-242) The oxygenase and reductase domains are joined by a calmodulin binding
5 peptide that regulates the activity of the NOS isozymes. Interestingly, NOS functions as a dimer. Reduction occurs in *trans*—the reductase domain from one monomer reduces the oxidase domain of the complementary monomer.(Crane, B. R. et al., *The EMBO Journal* (1999) **18**, 6271-6281)

10 The currently known mammalian NOS enzymes are organized into three classes: nNOS (neuronal), iNOS (immune), and eNOS (endothelial). These classifications reflect the origins of the NOS isoforms.(Bredt, D. S. et al., *Nature* (1991) **351**, 714-718; Janssens, S. P. et al., *J. Biol. Chem.* (1992) **267**, 22694; Lamas, S. et al., *Proc. Natl. Acad. Sci. USA* (1992) **89**, 6348-6352; Lowenstein, C. J. et al., *Proc. Natl. Acad. Sci. USA* (1992) **89**,
15 6711-6715; Xie, Q. W. et al., *Science* (1992) **256**, 225-228) However, subsequent research has shown that the various forms of NOS occur in a wide variety of tissues, with a complex distribution.

Although nNOS is constitutively expressed, its level of expression is dynamically
20 regulated.(Dawson, T. M. et al., *Progress in Brain Research* (1998) **118**, 3-11) For example, nNOS activity is high in the developing olfactory and visual systems, but low in their mature counterparts. Abnormal nNOS activity has been implicated in a variety of diseases, including both Parkinson's and Alzheimer's disease.(Luth, H. J. et al., *Brain Research* (2000) **852**, 45-55; Dawson, V. L.; Dawson, T. M. *Progress in Brain Research*
25 (1998) **118**, 215-229) The isozyme eNOS (endothelial NOS) is expressed in smooth muscles, including those lining blood vessels.(Huang, P. L. et al., *Nature* (1995) **377**, 239-242) Local production of NO triggers the relaxation of the vascular tissue, leading to reduction in blood pressure. In addition to vasodilation, eNOS also modulates angiogenesis.(Dimmeler, S.; Zeiher, A. M *Cell Death and Differentiation* (1999) **6**, 964-
30 968) iNOS has both beneficial and destructive influences in the immune system.(D. Nathan, *J. Clin. Invest.* (1997) **100**, 2417-2423) For instance, iNOS is thought to be

essential in fighting *Mycobacterium tuberculosis*.(MacMicking, J. D. et al., *Proc. Natl. Acad. Sci. USA* (1997) **94**, 5243-5248) However, iNOS is also involved in the often destructive inflammation response to infection or injury.(D. Nathan, *J. Clin. Invest.* (1997) **100**, 2417-2423)

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Despite the intense interest in nitric oxide synthases in the biological and medical community, aspects of the catalytic mechanism of these enzymes remain poorly understood. In particular, the function of the H₄B cofactor has not been adequately explained.

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Currently, two general methods are used for imaging NOS distribution.(Feelisch, M.; Stamler, J. S. Eds., *Methods in Nitric Oxide Research* (John Wiley and Sons, Inc., New York, 1996)) First, NADPH, arginine, NO, citrulline, nitrates, nitrates and other reactants and products of NOS can be detected chemically, usually through chemiluminescence or a stain (Kikuchi, K. et al., *Analytical Chemistry* (1993) **65**, 1794-1799; Kojima, H. et al., *Anal. Chem.* (1998) **70**, 2446-2453; Kishimoto, J. et al., *Eur. J. Neuroscience* (1993) **5**, 1684-1694). Because these small molecules diffuse rapidly, this limits the spatial resolution of this technique. In addition, the staining techniques kill the sample. The chemiluminescence resulting from the reaction of NO with ozone can also be used to quantify NO production, but again gives limited spatial information. The second technique used is immunohistochemistry (Maines, M. D Ed., *Nitric Oxide Synthase: Characterization and Functional Analysis* (Academic Press, San Diego, CA, 1996); Kobzik, L., Schmidt, H. H. H. W. in *Methods in nitric oxide synthase* M. Feelish, J. S. Stamler, Eds. (John Wiley and Sons, New York, 1996) pp. 229-236). Briefly, an antibody is raised against NOS. The antibody binds to NOS, and the antibody is then detected through staining or fluorescence. This gives better spatial resolution, but again the staining process destroys the sample.

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The disadvantages of many of the prior known probes utilized to study biomolecules are their requirements for chemical or biological modification of the biomolecules for characterization. Furthermore, because Ru-substrates interact with their targets

detected. The presence or absence of the detectable signal indicates whether the sensitizer-linked substrate and the biomolecule remain in close proximity to each other, indicating the modulation of activity by the candidate activity modulator.

- 5 Kits, compositions and integrated systems for performing the assays are also provided.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts the crystal structure of the P450_{cam}:Ru-C₉-Ad complex, as described in
 10 Example I, *infra*. The Ru-substrate is shown in yellow to highlight docking of {Ru(bpy)₃}²⁺ at the surface of the protein, as predicted by computer modeling and energy-transfer experiments.

Figure 2 shows the Ruthenium sensitizer-linked substrates, as described in Example I,
 15 *infra*.

Figure 3 illustrates the kinetics traces of [Ru-C₉-Ad]²⁺ emission decay at room temperature in solution and in a single crystal of P450_{cam}:Ru-C₉-Ad. [Ru-C₉-Ad]²⁺ (10 μM) exhibits monophasic decay (black) , as described in Example I, *infra*. Emission
 20 decay of [Ru-C₉-Ad]²⁺ equimolar with P450 (10 μM) is biphasic (red). In a P450:Ru-C₉-Ad crystal, {Ru(bpy)₃}²⁺ quenching is predominantly monophasic (blue).

Figure 4 shows a table of dissociation constants, Ru²⁺ excited-state lifetimes, and Ru-Fe distances, as described in Example I, *infra*.

25 Figure 5 depicts the specific detection of Cytochrome P450 by Ru-C₉-Ad, as described in Example I, *infra*.

Figure 6 depicts sensitizer-linked substrate, Ru-C₉-Ad, as described in Example II, *infra*.
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Figure 7 shows the crystal structure of the P450_{cam}:Ru-C₉-Ad conjugate, as described in Example II, *infra*. Although both Λ and Δ isomers are present, only Λ (magenta) is shown. The substituted bipyridyl ligand sits at the mouth of the cavity in close proximity to several hydrophobic residues, including Phe 193 and Tyr 29 (blue). The Ru-substrate amide carbonyl (red) hydrogen bonds to Tyr 96 (green).

Figure 8 illustrates the binding of a single substrate to P450, as described in Example II, *infra*.

Figure 9 shows the CD spectra of the enantiomeric forms of [Ru(bpy)₂(bpy-C₉-Ad)]²⁺: Δ (dotted line); Λ (solid line), as described in Example II, *infra*.

Figure 10 illustrates the inverse absorbance changes at 392 nm as functions of inverse camphor concentration on titrating camphor into buffered (50 mM potassium phosphate, 100 mM potassium chloride, pH 7.4) solutions of ferric-aquo cytochrome P450_{cam} (~5 μ M), as described in Example II, *infra*. The triangles denote the binding of camphor to P450 in the absence of any Ru-substrate (slope = $K_S/([P450]\Delta\epsilon$; $K_S = 3.0 \pm 0.2 \mu$ M, $[P450] = 5.50 \mu$ M, $\Delta\epsilon_{392 \text{ nm}} = 54,000 \text{ M}^{-1}\text{cm}^{-1}$). Inhibition of camphor binding by Λ -[Ru-C₉-Ad]Cl₂ (squares, $K_S = 74 \pm 10$, $[P450] = 5.15 \mu$ M, $\Delta\epsilon_{392 \text{ nm}} = 34,000 \text{ M}^{-1}\text{cm}^{-1}$ (difference between camphor- and [Ru-C₉-Ad]-bound P450), $[Ru] = 4.83 \mu$ M, $K_I = 15 \pm 1$, $K_D = 200 \pm 50 \text{ nM}$) and Δ -[Ru-C₉-Ad]Cl₂ (circles, $K_S = 53 \pm 10$, $[P450] = 5.29 \mu$ M, $[Ru] = 4.99 \mu$ M, $K_I = 10 \pm 1$, $K_D = 300 \pm 50 \text{ nM}$) is reflected by steeper slopes. Reported dissociation constants are averages of three titrations.

Figure 11 shows the absorbance (open circles, 392 nm; filled circles, 416 nm) versus Ru-C₉-Ad concentration, as described in Example II, *infra*; UV-vis data monitored the displacement of camphor from P450, and were corrected for Ru absorbance. Data are fit by the function: $A = A_0 + \Delta\epsilon*([P450]*[Ru])/([Ru] + K_S)$, where A_0 is the initial absorbance, $\Delta\epsilon_{392} = 34,000$, $\Delta\epsilon_{416} = 41,000 \text{ M}^{-1}\text{cm}^{-1}$, $[P450] = 4.67 \mu$ M; $K_S(392 \text{ nm}) = 13.3$, $K_S(416 \text{ nm}) = 11.7$. $K_D = K_{\text{cam}}/K_S = 240 \pm 20 \text{ nM}$.

Figure 12 depicts the kinetics traces of $[\text{Ru-C}_9\text{-Ad}]^{2+}$ emission decay, as described in Example II, *infra*. A larger fraction of $\Lambda\text{-}[\text{Ru-C}_9\text{-Ad}]\text{Cl}_2$ emission (monitored at 620 nm) is quenched by P450.

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Figure 13 shows the 1/fraction of quenched Ru^{2+} luminescence and 1/fraction of low-spin P450 as functions of camphor concentration, as described in Example II, *infra*.

Figure 14. Ru-compounds (1) and (3) for the study of P450 catalysis in an open conformation. While (1) competes with camphor for the P450 active site, (2) is able to share the pocket with camphor. 2-adamantylacetamide (2) is analogous to compound (1), without the Ru-tether; (2) induces a full low to high spin conversion at the heme.

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Figure 15. ESI mass spectra showing the doubly charged starting material, $[\text{Ru-C}_9\text{-Ad}]^{2+}$ barely visible on the left, and the conversion to products, $[\text{Ru-C}_9\text{-Ad-OH}]^{2+}$, on the right.

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Figure 16. Calibration line showing the relationship between the ratio of $[\text{Ru-C}_9\text{-Ad}]^{2+} / [\text{Ru-C}_9\text{-Ad-OH}]^{2+}$ in solution to the relative ionization intensity of both cations in the ESI.

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Figure 17. Variation of the resonance Raman $\text{Fe}^{2+}\text{-(CO)}$ stretching mode with substrate. Spectra for $\text{Ru-C}_{11}\text{-Ad}$, $\text{Ru-C}_9\text{-Ad}$, and adamantane differ little, varying by only a few cm^{-1} in their peak maximum.

Figure 18. A table of substrate Analog Induced Changes of the CO-Stretching Mode in P450

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Figure 19. Top: Rate of NADH consumption in turnover studies of $\text{Ru-C}_9\text{-Ad}$. The slope indicates a rate of 8 $\mu\text{mol NADH/min}/\mu\text{mol P450}$. Bottom: Control experiment showing the rate of NADH consumption ($\sim 30 \mu\text{mol NADH/min}/\mu\text{mol P450}$) in the absence of any substrate.

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Figure 20. Electrospray mass spectrum showing a possible oxidized Ru-C₉-Ad product isolated after photolysis of the P450:Ru-C₉-Ad complex. Zooming in on this species shows it is doubly charged and has a similar profile to the spectrum of hydroxylated Ru-C₉-Ad, shown in Figure 4.4.

Figure 21. A two substrate, ternary binding model for the P450 (E):camphor (C): Ru-C₁₀ (R) system.

Figure 22. The coefficients corresponding to the three different states of Ru (free, bound to P450 alone, or bound in the ternary complex) as a function of camphor concentration. The rate of decay, k_d , of unbound Ru^{2+*} in aerated solution was determined: $k_d = 2.5 \times 10^6 \text{ s}^{-1}$. The rate of Ru^{2+*} decay when bound to P450 was found to be $k_{P450} = 9.8 \times 10^6 \text{ s}^{-1}$. Finally, the Ru^{2+*} emission is considerably longer-lived in the ternary complex, decaying with the rate constant $k_{\text{tern}} = 7.8 \times 10^6 \text{ s}^{-1}$. The relative percentage of each component can be calculated by dividing one coefficient by the sum of all of the coefficients.

Figure 22A. Top: Rate of NADH consumption in the complex between P450 (1 μM) and camphor (1 mM). The initial decay rates were typically $\sim 600 \mu\text{mol NADH/min}/\mu\text{mol}$ P450. Bottom: Rate of NADH consumption in the complex between P450 (1 μM), camphor (100 μM) and Ru-C₁₀ (10 μM). The initial decay rates were typically $\sim 300 \mu\text{mol NADH/min}/\mu\text{mol}$ P450.

Figure 23 depicts the oxidative flash-quench scheme by which HRP is oxidized to the compound I state, as described in Example IV, *infra*. These chemistries, and the oxidation states shown, serve as a model for possible high-valent intermediates in oxygenases, such as P450.

Figure 24 shows the sensitizer-linked substrates (Ru-Ad, Ru-EB) and ligands (Ru-Im) for photooxidation and reduction of P450, as described in Example IV, *infra*.

Figure 25 is a scheme of the BILRC nanosecond experiment table used to collect full spectrum transient absorption data with a diode array detector, as described in Example IV, *infra*. Probe light from the flash lamp is sent via a fiber optic through a beam splitter and focused onto separate reference and sample fiber optics.

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Figure 26 shows a single-wavelength transient absorption spectra, as described in Example IV, *infra*: Δ -absorbance versus time plots for the reaction of $[\text{Ru-Im}]^+$ with P450. Changes in the Soret region (bleach of $\text{Fe}^{3+}\text{-Im}$ at 420 nm and increase of $\text{Fe}^{2+}\text{-Im}$ at 445 nm) were observed after laser excitation of a 10 μM P450, 10 μM $\text{Ru-C}_{13}\text{-Im}$, 10 mM *p*-MDMA sample.

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Figure 27 depicts the diode array spectra of P450 at various time delays during and after photoreduction by $[\text{Ru-EB}]^+$, as described in Example IV, *infra*. The broad, sloping intensity at 350 nm at 14 μs can be assigned to spectral contributions from $[\text{Ru-EB}]^+$ and *p*-MDMA.

15

Figure 28 illustrates the single-wavelength transient absorption spectra, as described in Example IV, *infra*: Δ -absorbance versus time plots for the reaction between $\text{Fe}^{3+}\text{-aquo}$ P450 ($\lambda_{\text{max}} = 417 \text{ nm}$) and $[\text{Ru-EB}]^{3+}$. The photooxidation product, centered at 390 nm, was observed with the same kinetics as the disappearance of the starting species at 417 nm. Samples were 10 μM P450, 10 μM Ru-EB , and 5 mM $[\text{Co}(\text{NH}_3)\text{Cl}]^{2+}$.

20

Figure 29 shows the diode array spectra of P450 showing photooxidation by $[\text{Ru-EB}]^{3+}$ 1 ms after laser excitation, as described in Example IV, *infra*. The absorbance changes are due mostly to oxidation of P450. Oxidized spectra of WT P450 and Y96F are fairly similar in profile, but differ in intensity; higher yields in the mutant enzyme suggest that tyrosine intercepts some of the Ru^{3+} before it oxidizes the heme.

25

Figure 30 depicts the proposed flash-quench scheme for generating P450 high-valent intermediates by both oxidative and reductive chemistries, as described in Example IV, *infra*.

30

Figure 31 illustrates the overall flash/quench reaction scheme showing the preparation of new redox states in the P450:Ru-EB complex, as described in Example IV, *infra*. Reversible electron-transfer processes return Ru and P450 to their resting states within 100 ms of the initial laser pulse.

Figure 32 displays the structures for the compounds, as described in Example V, *infra*. Top: Three conjugated sensitizer-linked probes in their presumed orientation relative to cytochrome P450_{cam} (thiolate-ligated heme). Bottom: Model compounds (d) and (e) for electrochemical studies and control experiments involving transient absorption spectroscopy.

Figure 33 depicts the table of K_D , k_{ET} , k_{en} , R_0 , and Ru-Fe distances of Ru-probes (a-c)*, as described in Example V, *infra*.

Figure 34 depict the spectra, as described in Example V, *infra*. Top: Q-bands of P450 bound to (a) (substrate-free spectrum), (b), and (c). The spectral overlap of the Ru^{2+*} emission with the Q-band absorption gives the Förster distance. Below: Emission spectra of [Ru(bpy)₃]^{2+*}, Ru-Im (b), and tmRu-Im (a, c). Integration of the area under each spectrum and comparison to a standard [Ru(bpy)₃]²⁺ solution gave the quantum yields as described in Appendix D. Q.Y. (Ru(bpy)₃²⁺) = 0.042, Q.Y. (Ru-Im, (b)) = 0.025, Q.Y. (tmRu-Im, (a, c)) = 0.0094.

Figure 35 illustrates single-wavelength transient absorption, as described in Example V, *infra*: Δ -absorbance versus time plots for the reaction of [Ru-biphenF₈-Im]⁺ with P450. Changes in the Soret region (bleach of Fe³⁺-Im at 420 nm and increase of Fe²⁺-Im at 445 nm) were observed after laser excitation of a 5.3 μ M P450, 5.3 μ M Ru sample.

Figure 36 depicts transient absorption of the P450:Ru-biphenF₈-im complex, collected 5 μ s after laser excitation, as described in Example V, *infra*. Δ Absorption intensities were obtained by fitting transient absorption kinetics for several different wavelengths in the

Soret region. At 5 μ s, all of the Ru^{2+*} has been consumed, and the observed difference spectrum is a sum of the absorption changes caused by (P450) Fe^{2+} -Im and Ru^{3+} .

Figure 37 depicts luminescence decay profile (620 nm) for tmRu-biphenF₈-im^{2+*} both free in solution (monophasic) and bound to P450 (biphasic), as described in Example V, *infra*..

Figure 38 illustrates transient absorption kinetics profile of P450 bound to (c), collected at 445 nm (top) and 420 nm (bottom). $[\text{P450}] = [\text{Ru}] = 11 \mu\text{M}$, laser power = 3.3 mJ/pulse, as described in Example V, *infra*..

Figure 39 depicts the absorption spectra, as described in Example V, *infra*. Top: Transient absorption of the excited state of Ru-biphenF₈-im, collected 10 ns after laser excitation ($[\text{Ru}] = 8.9 \mu\text{M}$, laser power = 3.3 mJ/pulse). Bottom: Transient absorption of the P450:Ru-biphenF₈-im complex, collected 30 ns after laser excitation ($[\text{P450}] = [\text{Ru}] = 8.9 \mu\text{M}$, laser power = 3.3 mJ/pulse). Δ Absorption intensities were obtained by fitting transient absorption kinetics for several different wavelengths in the Soret region. At 30 ns, the observed difference spectrum is a sum of the absorption changes caused by Ru^{2+*} (shown above, $\Delta\epsilon \text{ Ru}^{2+*} - \text{Ru}^{2+} = -2400 \text{ M}^{-1}\text{cm}^{-1}$ at 445 nm, $-1500 \text{ M}^{-1}\text{cm}^{-1}$ at 420 nm), Ru^{3+} ($\Delta\epsilon \text{ Ru}^{3+} - \text{Ru}^{2+} = -9000 \text{ M}^{-1}\text{cm}^{-1}$ at 445 nm, $-6000 \text{ M}^{-1}\text{cm}^{-1}$ at 420 nm), and P450 Fe^{2+} -Im ($\Delta\epsilon \text{ Fe}^{2+} - \text{Fe}^{3+} = 81,000 \text{ M}^{-1}\text{cm}^{-1}$ at 445 nm, $-82,000 \text{ M}^{-1}\text{cm}^{-1}$ at 420 nm, calculated from the spectra shown in Figure 37).

Figure 40 shows the UV-vis absorption spectra of tmRu-biphenF₈-Im (c) alone and complexed with both ferric and ferrous P450; all species are 5.2 μM , as described in Example V, *infra*.. The Fe^{2+} -Im spectrum is not shown below 400 nm, because dithionite dominates the absorbance in this region. Extinction coefficients at the absorbance maxima of ferric ($\epsilon_{420} = 100,000 \text{ M}^{-1}\text{cm}^{-1}$) and ferrous ($\epsilon_{445} = 103,000 \text{ M}^{-1}\text{cm}^{-1}$) are quite similar. tmRu-biphenF₈-Im has an absorbance maximum at 444 nm ($\epsilon_{444} = 14,500 \text{ M}^{-1}\text{cm}^{-1}$). In the ferrous state, the P450 Q-bands coalesce and gain intensity (ferric, $\epsilon_{540} =$

11,000 M⁻¹cm⁻¹, ε₅₆₈ = 10,000 M⁻¹cm⁻¹; ferrous, ε₅₄₆ = 12,700 M⁻¹cm⁻¹), as is typical for P450:imidazole complexes.

Figure 41 depicts the power dependence of the ΔOD at 445 nm ($[P450] = [tmRu-$
 5 biphenF₈-Im] = 10 μM). Saturation occurs ~ 3.3 mJ/pulse, showing that nearly 100% of
 the Ru has been excited.

Figure 42 displays the diode array spectra of direct photoinduced reduction of the (P450) Fe^{3+} -im-biphenF₈-Rutm complex at various time intervals after laser excitation, as described in Example V, *infra*.. The reduced enzyme returns to the ferric resting state by 10 three different channels, with some of the ET products persisting for up to a second.

Figure 43 illustrates the overall electron-transfer scheme for photoreduction of the P450:[Ru(tmbpy)₂(bpy-biphenF₉-im)]Cl₂ complex, as described in Example V, *infra*..

15 Rates of energy transfer and electron transfer are based on the calculated yields of the reduced (Fe²⁺-im) enzyme (see text for details). For this complex, the dominant decay channel for Ru²⁺ is ET, presumably due to the fully conjugated path to the heme, and the extra driving force for reduction provided by the tetramethylated bipyridyl ligands. Highly exergonic back electron transfer occurs rapidly from Fe²⁺-im to Ru³⁺. Competing

20 with this process is believed to be oxidation of Tyr 29 which sits in close proximity (~ 3 Å) to the Ru moiety, and allows the reduced protein to persist for tens of microseconds. A third back ET pathway (not shown) must involve scavenging of the tyrosyl radical, either by oxidation of exogenous Ru²⁺ or other reductants in solution. This bimolecular process allows a small fraction (~ 5%) of the reduced, imidazole-bound protein to persist

25 for 1 second in solution.

Figure 44 shows the cyclic voltammogram on an edge-plane graphite electrode of tmRu (e) in acetonitrile at a scan rate of 100 mV s^{-1} at 298 K, as described in Example V, *infra*.

Figure 45 illustrates the cyclic voltammogram on an edge-plane graphite electrode of tmRu-biphenF₈-im in acetonitrile at a scan rate of 100 mV s⁻¹ at 298 K, as described in Example V, *infra*..

5 Figure 46 depicts the compounds as described in Example VI, *infra*.

Figure 47 shows the synthetic scheme of compound 3, as described in Example VI, *infra*.

10 Figure 48 illustrates compound 3 docked in the open conformation of P450, as described in Example VI, *infra*. The original Ru^{II}(bpy)₃ complex is shown for reference.

Figure 49 shows the luminescence of 3 in 10 μM 3 in buffer, in buffer with P450 and in acetonitrile, as described in Example VI, *infra*.

15 Figure 50 depicts how the alkyl chain will fold back ideally, filling the channel and protecting the dppz from water, as described in Example VI, *infra*.

Figure 51 illustrates the fluorophore classes A (7-amino-4-methyl-6-sulfocoumarin-3-acetamide) and B (7-methoxycoumarin-3-acetamide), as described in Example VII, *infra*.

20

Figure 52 depicts luminescent probes, as described in Example VII, *infra*.

Figure 53 shows the excited state processes of Ru^{II}(bpy)₃, as described in Example VII, *infra*.

25

Figure 54 illustrates the luminescent probe classes C and D, as described in Example VIII, *infra*.

30 Figure 55 depicts the series of molecules synthesized by Yonemoto *et al.* (Yonemoto, E. H. et al., *J. Am. Chem. Soc.* (1994) 116, 4786-4795), as described in Example VIII, *infra*.

Figure 56 depicts the synthesis of the class D probes, as described in Example VIII, *infra*.

DETAILED DESCRIPTION OF THE INVENTION

5 The subject invention is a modular approach to generating the sensitizer-linked substrate molecules. These molecules are useful, e.g., in methods for detection of target biomolecules. These detection methods in which sensitizer-linked substrates assess ligand specificity and enzyme structure are (1) superior to existing enzyme- and antibody-based assays; and (2) are amenable to combinatorial chemistry. In addition,
10 enantiospecific interactions may be exploited in the design of enzyme-metallosubstrate conjugates using the invention.

Definitions

15 As used in this application, the following words or phrases have the meanings specified.

As used herein a "target biomolecule" is any protein that can be targeted with a high affinity molecule (i.e. binding element or substrate) specific for that protein. Such biomolecules include, but are not limited to, enzymes such as oxidases, reductases,
20 synthases, synthetases, kinases, phosphatases, G proteins, membrane proteins, receptor proteins, and ion channels. The biomolecule may or may not contain a chromophore.

As used herein a "sensitizer-linked substrate" molecule is a compound having a substrate (i.e. binding element) attached by a linker (i.e. tether) to a sensitizer element.

25 As used herein a "sensitizer" is an element (i.e. moiety, group, label, or tag) that can emit energy, e.g., as luminescence through photochemical, chemical, and/or electrochemical processes. Photoluminescence can be defined as a process whereby a material is induced to luminesce when it absorbs electromagnetic radiation. Fluorescence and
30 phosphorescence are forms of photoluminescence.

As used herein a "substrate" is a binding element, compound, or molecule that has a high affinity and/or high specificity of binding for the target biomolecule.

5 As used herein a "linker" is a molecule of a length sufficient to allow the substrate to bind to the active site of the biomolecule so that upon binding the sensitizer is located within the protein or near the protein surface.

10 As used herein a "modulator" is any agent that can alter the activity of a target biomolecule, the agent can be a small organic molecule, protein or protein fragment, nucleic acid.

In order that the invention herein described may be more fully understood, the following descriptions are set forth.

15 **Compositions of the Invention**

The present invention is directed to the detection and characterization of target biomolecules by novel sensitizer-linked substrate molecules. The invention provides for novel sensitizer-linked substrate molecules having a sensitizer, a linker, and a substrate
20 for use in detecting and characterizing target biomolecules. The sensitizer-linked substrate molecules are highly specific, selective, and have high affinity for its target biomolecule and the signal emitted by these molecules allows detection at low concentrations.

25 A preferred embodiment of a target biomolecule is a protein that possesses natural or unnatural cofactors that would quench sensitizers or modifies the signal of the molecule of the invention which binds to its target.

30 Such target biomolecules having a metal chromophore are termed metalloproteins. Such metalloproteins include, but are not limited to, Cytochrome P450, Superoxide Dismutase

(SOD), Nitric Oxide Synthase (NOS), heme oxygenase, prostaglandin H synthase, soluble guanylylate cyclase, prostacyclin synthase, and amine oxidases.

5 Examples of Cytochrome P450 include, but are not limited to, 1A1, 1A2, 2A6, 2C8, 2C9, 2D6, 2E1, 3A4, 3A5, and 4A11.

10 Examples of NOS include, but are not limited to, NOS isozymes are iNOS (inducible), nNOS (neuronal), and eNOS (endothelial), but the method of the invention could also be extended to the discovery of new NOS isozymes (Feelisch, M.; Stamler, J. S. Eds., *Methods in Nitric Oxide Research* (John Wiley and Sons, Inc., New York, 1996)).

15 Target biomolecules include those having a flavin chromophore such as FMN or FADH. Examples of flavoproteins include, but are not limited to P450 reductase, D-amino-acid oxidase and flavocytochrome b2.

20 Types of substrates that have a high affinity for target biomolecules include, but are not limited to, molecules that bind to the active site, recognition, allosteric, or cofactor site of the biomolecule, such that the substrate molecule can bind in the selected site and occupy the cavity or space, or that bind to the active site and undergo transformation of the substrate, the biomolecule, and/or both the substrate and biomolecule.

25 Substrates are any agents that can bind to the target biomolecule. Substrates can include activators or inhibitors of the target biomolecule. Substrates of the invention include, but are not limited to, small organic molecules, protein fragments, nucleic acid molecules, and/or CDR of antibodies.

30 Substrates of Cytochrome P450 include, but are not limited to, caffeine, testosterone, progesterone, ethylmorphine, aminopyrine, benzphetamine, 7-ethoxycoumarin, warfarin, ethylbenzene, adamantane, carbon monoxide, metyrapone, allylisopropylacetamide, and imidazole and its derivatives.

Substrates of NOS include, but are not limited to, N^G-monomethyl, dimethyl, nitro, and amino arginines, N^G-nitro-L-arginine methyl ester, N^δ-(iminoethyl-L-ornithine, L-thiocitrulline, S-alkyl-L-thiocitrulines, bithioureas, 7-nitroindazoles, aminoguanidine, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine, 2-iminoazaheterocycles, N-phenylisothioureas, N-phenylamidines, nitroaromatic amino acids and modifications of these compounds. (Collins, J.L. et al., *J. Med. Chem.* (1998) **41**, 2858-2871; Hibbs, J.B. et al. *J. Immunol.* (1987) **138**, 550-565; Lamber, L.E. et al., *Life Sci.* (1991) **48**, 69-75; Rees, D.D. et al. *Br. J. Pharmacol.* (1990) **101**, 746-752; Gross, S.S. et al., *Biochem. Biophys. Res. Commun.* (1990) **270**, 96-203; Furfine, E.S. et al., *Biochemistry* (1993) **32**, 8512-8517; Narayanan, K. et al., *J. Med. Chem.* (1994) **37**, 885-887; Narayanan, K. et al., *J. Biol. Chem.* (1995) **270**, 11103-11110; Furfine, E.S. et al., *J. Biol. Chem.* (1994) **269**, 26677-26683; Garvey, E.P. et al., *J. Biol. Chem.*, (1994) **269**, 26669-26676; Wolff, D.J. et al., *Arch. Biochem. Biophys.* (1994) **311**, 300-306; Hasan, K. J. et al., *Pharmacol.* (1993) **249**, 101-106; Moore, W.M. et al., *J. Med. Chem.* (1996) **39**, 669-672; Moore, W.M. et al., *Bioorg. Med. Chem.* (1996) **4**, 1559-1564; Shearer, B.G. et al., *J. Med. Chem.*, (1997) **40**, 1901-1905; Garvey, E.P. et al., *J. Biol. Chem.* (1997) **272**, 4959-4963. Cowart, M. et al., *J. Med. Chem.* (1998) **41**, 2636-2642).

Types of linkers that can be used to connect the sensitizer to the substrate include substituted or unsubstituted, cyclic or acyclic alkyl, alkene, alkynyl, alkoxy chains, or aryl groups, extended fused aromatic ring systems (e.g. naphthalene, anthracene), peptides, ethers, thioethers, esters, amines, amides and oligomers thereof.

The length of the linker is designed to be sufficient to allow the substrate to interact favorably with the target biomolecule and that upon binding, the sensitizer is located within the biomolecule or near the biomolecule surface. The distance between the sensitizer and the biomolecule can be varied depending on the site to which the sensitizer-linked substrate molecule binds, e.g. active site, recognition, allosteric, or cofactor site of the biomolecule. For example, the distance can be less than or equal to 100 Å.

A linker can be tailor made to enhance properties of the sensitizer-linked substrate molecules such as hydrophobicity and cellular uptake, and may be readily modified to form improved sensitizer-linked substrate molecules for use as diagnostic and therapeutic agents. Where electron transfer is not a factor, any linkage of sufficient length, hydrophobicity, and charge will be useful in the methods of the invention. In an embodiment of the invention, where the linker is a methylene chain, the length of the linker between the sensitizer and the substrate can be varied for $(CH_2)_n$ where n is 1 to 13.

Types of sensitizers that can emit energy as luminescence include both organic and inorganic photosensitizers (Erkkila, K.E. et al., *Chem Rev.* 1999, 99, 2777-2795) and fluorophores.

Photosensitizers for use in the invention include, but are not limited to, $[Ru(bpy)_3]^{2+}$, $[Ru(phen)_2dppz]^2$, $[Ru(bpy)CN_4]^{2-}$, $[Os(tpy)_2]^{2+}$, where tpy is 2,2':6',2"-terpyridine, $[Os(ttpy)_2]^{2+}$, where ttpy is 4'-(p-tolyl)-2,2':6',2"-terpyridine, $[Os(tptpy)_2]^{2+}$, where tptpy is 4,4',4"-triphenyl-2,2':6',2"-terpyridine (M. Beley, J.-P. Collin, J.-P. Sauvage, H. Sugihara, F. Heisel, A. Mische, *J. Chem. Soc. Dalt.* 1991, 3157-3159.), as well as $[Os(phen)_3]^{2+}$ and $[Os(phen)_2(Me_2PhP)_2]^{2-}$ (E.M. Kober, et al., *J. Am. Chem. Soc.*, 1980, 102, 7383-7385; E.M. Kober et al., *Inorg. Chem.* 1985, 24, 2755.), or where applicable, a geometric or optical isomer or racemic mixture thereof.

Sensitizers can include luminescent metal complexes. Luminescent metal complexes include, but are not limited to, homo- and heteroleptic ruthenium terpyridine, bipyridine, pyridine, imidazole, cyano and carbonyl complexes, as well as complexes of other transition metals, including but are not limited to osmium, platinum, iridium, rhenium, rhodium, molybdenum, tungsten and copper (Roundhill, D.M. *Photochemistry and Photophysics of Metal Complexes* (Plenum Press, New York, 1994; Horvath, O. and Stevenson, K.L. *Charge Transfer Photochemistry of Coordination Compounds* (VCH Publishers, Inc., New York, 1992).

Luminescence compounds include, but are not limited to, methyl viologens, quinones, N,N-dialkylanilines, N,N-dialkyl-p-methoxyanilines and triarylaminines. [Horvath, O. and Stevenson, K.L. *Charge Transfer Photochemistry of Coordination Compounds* (VCH Publishers, Inc., New York, 1992)].

5

Types of organic fluorophores include, but are not limited to, coumarins, Texas red, 1- and 2-aminonaphthalenes, p,p'-diaminostilbenes, pyrenes, anthracenes, fluoresceins, and rhodamines.

- 10 Types of sensitizer-linked substrate molecules of the invention include, but are not limited to, $[\text{Ru-C}_{13}\text{-EB}]^{2+}$, $[\text{Ru-C}_{12}\text{-EB}]^{2+}$, $[\text{Ru-C}_{11}\text{-EB}]^{2+}$, $[\text{Ru-C}_{10}\text{-EB}]^{2+}$, $[\text{Ru-C}_9\text{-EB}]^{2+}$, $[\text{Ru-C}_7\text{-EB}]^{2+}$, $[\text{Ru-C}_{11}\text{-Ad}]^{2+}$, $[\text{Ru-C}_9\text{-Ad}]^{2+}$, $[\text{Ru-C}_{13}\text{-Im}]^{2+}$, $[\text{Ru-C}_{13}\text{-Im}]^{2+}$, $[\text{Ru-dppa-C}_6\text{-Ad}]$, $[\text{Ru-dppa-gly-Ad}]$, and $[\text{Ru-dppa-Ad}]$. Where, Ru is $[\text{Ru}(\text{bpy})_3]^{2+}$ complex, Ru-dppa is $[\text{Ru}(\text{phen})_2\text{dppa}]^{2+}$ complex, the C_n is a methylene chain of n carbon atoms long,
- 15 EB is ethylbenzene, Ad is adamantane, and Im is imidazole.

Methods of the Invention

- In addition, the invention provides novel methods for detecting and characterizing target
- 20 biomolecules using the sensitizer-linked substrate molecules of the invention.

- Characterization of biomolecules includes, but is not limited to, measurement of structural properties of the biomolecule such as the active site size, shape, and volume, aspects of substrate specificity, elucidation of the mechanism of action of the
- 25 biomolecule, and interactions between biomolecules, i.e. regulation or modulation of the biomolecule, especially by other biomolecules.

- The methods of detection and characterization of a biomolecule by a sensitizer-linked substrate molecules are performed by contacting a biomolecule with a sensitizer-linked
- 30 substrate molecule designed to interact with said biomolecule, under suitable conditions, and measuring a detectable signal for the interaction, e.g., a signal by the free sensitizer-

linked substrate molecule and/or a signal resulting from a combination of the biomolecule and sensitizer-linked substrate. The presence or absence of the detectable signal indicates whether the sensitizer-linked substrate and the biomolecule remain in close proximity to each other.

5

In one embodiment of the method of the invention, the target biomolecule is Cytochrome P450, and the sensitizer-linked substrate molecules of the invention are selected from the group comprising $[\text{Ru-C}_{13}\text{-EB}]^{2+}$, $[\text{Ru-C}_{12}\text{-EB}]^{2+}$, $[\text{Ru-C}_{11}\text{-EB}]^{2+}$, $[\text{Ru-C}_{10}\text{-EB}]^{2+}$, $[\text{Ru-C}_9\text{-EB}]^{2+}$, $[\text{Ru-C}_7\text{-EB}]^{2+}$, $[\text{Ru-C}_{11}\text{-Ad}]^{2+}$, $[\text{Ru-C}_9\text{-Ad}]^{2+}$, $[\text{Ru-C}_{13}\text{-Im}]^{2+}$, $[\text{Ru-C}_{13}\text{-Im}]^{2+}$, $[\text{Ru-dppa-C}_6\text{-Ad}]$, $[\text{Ru-dppa-gly-Ad}]$, and $[\text{Ru-dppa-Ad}]$. Where, Ru is $[\text{Ru}(\text{bpy})_3]^{2+}$ complex, Ru-dppa is $[\text{Ru}(\text{phen})_2\text{dppa}]^{2+}$ complex, the C_n is a methylene chain of n carbon atoms long, EB is ethylbenzene, Ad is adamantane, and Im is imidazole.

10

Isolation and purification of human Cytochrome P450s is very difficult as these enzymes are generally membrane-bound. The method of the invention for detecting P450s in solution offers several advantages over traditional isolation protocols. First, where Ru sensitizers are used, due to the tight binding of Ru-linker-substrates, it is possible to work with cytochrome P450s at low concentrations where most isozymes should be soluble. Additionally, due to the sensitivity of the fluorescence technique, lower concentrations of P450 can be used to perform inhibitor binding studies or in assays to detect specific P450 isozymes. The potential for isozyme-specific binding may minimize the need for extended purification protocols in studies involving, a system such as the liver, where numerous P450 isozymes are present.

20

Fluorescence quenching may be used to determine biomolecule dimerization or protein-protein interactions. Alternatively, as has been shown extensively in work with blue and green fluorescent protein (GFP), fluorescence resonance energy transfer (FRET) may be employed to quantify distances between biomolecules. (A. Miyawaki, J. Llopis, R. Heim, J.M. McCaffery, J.A. Adams, M. Ikura, R.Y. Tsien, Nature 1997, 388, 882-887.) Upon excitation, the blue fluorophore transfers its energy to the green fluorophore. (R. Heim and R.Y. Tsien, Curr. Biol. 1996, 6, 178-182.) Quantifying the intensity, decay kinetics,

30

or polarisation of the emission by the green fluorophore provides a "molecular yardstick" by which to measure the separation between fluorophores. For example, a sensitizer-linked substrate attached to biomolecule X could identify interactions with biomolecule Y, provided that Y possesses a fluorophore that is sufficiently in resonance (overlap of absorption and emission profiles) with the sensitizer-linked substrate.

In cases where the target biomolecule does not contain a suitable chromophore (e.g., heme, flavin) to interact with the sensitizer, structural characterization may be achieved by labeling the biomolecule with a commercially available fluorescent probe (i.e. dansyl probes synthesized by Molecular Probes, Eugene, OR). Binding of a sensitizer-linked substrate to the labeled biomolecule is assessed by fluorescence quenching experiments as described for P450.

Methods of Screening

Also included within the scope of methods of detection of the invention are screening assays for detecting and identifying potential modulators of target biomolecules. These assays involve incubating a test mixture that contains a target biomolecule, a sensitizer-linked substrate molecule, and a candidate activity modulator, under conditions suitable for biomolecule activity. The presence or absence of a detectable signal, e.g., a signal by the free sensitizer-linked substrate molecule and/or a signal resulting from a combination of the biomolecule and sensitizer-linked substrate are detected. The presence or absence of the detectable signal indicates whether the sensitizer-linked substrate and the biomolecule remain in close proximity to each other.

In an embodiment of the methods of the invention, the target biomolecule and sensitizer-linked substrate can be complexed and placed together in a series of isolated compartments, such as those found on microtiter plates or pico- nano- or micro-liter arrays. Concentrations of the biomolecules and sensitizer linked substrate can be as low as their dissociation constant for each other ($< 10^{-5}$ M). Each well can be individually assessed for changes in luminescence intensity by automated laser confocal fluorescence

scanning (Fodor S., et al. Nature (1993) 364 555-556, Duggan D.J., et al (2000) Nature Genetics 21 suppl, 10-14) or by screening an entire set of wells by digital imaging (Joo H. et al., Nature (1999) 399, 670-673). Upon introduction of different candidate inhibitors for the target biomolecule to each compartment or well, changes in
5 luminescence of the sensitizer indicates successful competition between the biomolecule and the modulator.

The methods of the invention also include high throughput screening of large chemical libraries, e.g. by automating the assay steps and providing compounds from any
10 convenient source to assay. The assays are typically run in parallel (e.g., in microtiter formats on microtiter plates in robotic assays).

The high throughput screening methods of the invention involve providing a combinatorial library containing a large number of potential therapeutic compounds
15 (potential modulator compounds) (Borman, S, C. & E. News, 1999, 70(10), 33-48). Such "combinatorial chemical libraries" are then screened in one or more assays, as described herein, to identify library members (particular chemical species or subclasses) that display the ability to modulate the target biomolecule activity (Borman, S., *supra*; Dagani, R. C. & E. News, 1999, 70(10), 51-60). The compounds thus identified can
20 serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of
25 chemical "building blocks," such as reagents. For example, a linear combinatorial chemical library, such as a polypeptide library, is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building
30 blocks.

Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (see, e.g., U.S. Pat. No. 5,010,175, Furka, *Int. J. Pept. Prot. Res.*, 1991, 37:487-493 and Houghton, et al., *Nature*, 1991, 354, 84-88). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to, peptoids (PCT Publication No. WO 91/19735); encoded peptides (PCT Publication WO 93/20242); random bio-oligomers (PCT Publication No. WO 92/00091); benzodiazepines (U.S. Pat. No. 5,288,514); diversomers, such as hydantoins, benzodiazepines and dipeptides (Hobbs, et al., *Proc. Nat. Acad. Sci. USA*, 1993, 90, 6909-6913); vinylogous polypeptides (Hagihara, et al., *J. Amer. Chem. Soc.* 1992, 114, 6568); nonpeptidal peptidomimetics with .beta.-D-glucose scaffolding (Hirschmann, et al., *J. Amer. Chem. Soc.*, 1992, 114, 9217-9218); analogous organic syntheses of small compound libraries (Chen, et al., *J. Amer. Chem. Soc.*, 1994, 116, 2661; Armstrong, et al. *Acc. Chem. Res.*, 1996, 29, 123-131); or small organic molecule libraries (see, e.g., benzodiazepines, Baum *C&E News*, 1993, Jan. 18, page 33,); oligocarbamates (Cho, et al., *Science*, 1993, 261, 1303); and/or peptidyl phosphonates (Campbell, et al., *J. Org. Chem.* 1994, 59, 658); nucleic acid libraries (see, Seliger, H et al., *Nucleosides & Nucleotides*, 1997, 16, 703-710); peptide nucleic acid libraries (see, e.g., U.S. Pat. No. 5,539,083); antibody libraries (see, e.g., Vaughn, et al., *Nature Biotechnology*, 1996, 14(3), 309-314 and PCT/US96/10287); carbohydrate libraries (see, e.g., Liang, et al., *Science*, 1996, 274, 1520-1522 and U.S. Pat. No. 5,593,853, Nilsson, UJ, et al., *Combinatorial Chemistry & High Throughput Screening*, 1999 2, 335-352; Schweizer, F; Hindsgaul, O. *Current Opinion In Chemical Biology*, 1999 3, 291-298); isoprenoids (U.S. Pat. No. 5,569,588); thiazolidinones and metathiazanones (U.S. Pat. No. 5,549,974); pyrrolidines (U.S. Pat. Nos. 5,525,735 and 5,519,134); morpholino compounds (U.S. Pat. No. 5,506,337); benzodiazepines (U.S. Pat. No. 5,288,514); and other similar art.

For high-throughput screening, the number of potential inhibitors tested are further increased by combining an expanded multiple array format with light-directed combinatorial chemical synthesis and laser confocal fluorescence scanning (Fodor S. et al, *Science* (1991) 251, 767-773; Fodor S., et al. *Nature* (1993) 364 555-556; Cho C. et

al, Science (1993) 261, 1202-1305; Fodor S., et al. Nature (1993) 364 555-556; Rozsnyai L., Angew. Chem. (1992) 31, 759-761; McGall G. et al, Proc. Natl. Acad. Sci. USA (1996), 93, 13555-13560; McGall G. et al., J. Am. Chem. Soc. (1997) 119, 5081-5090. For example, light-directed combinatorial chemistry is used to generate a library of
5 potential inhibitors adhered in a 2-dimensional microarray format on a solid glass surface.

Alternatively, robotic spotting of an inhibitor library on a surface may be used to generate the array (Cheung V.G. et al, (2000) Nature Genetics 21 suppl, 15-19). In this
10 application, every inhibitor can contain within it a chemical moiety for bonding to the surface and an appropriately linked sensitizer element capable of responding to a chromophore(s) contained in the biomolecule to be assayed. The substrate (i.e. recognition element) directed at the biomolecule and linkage to the sensitizer can be varied combinatorially. Thus, each position on the grid can include a sensitizer-linked
15 substrate each containing a different substrate moiety for recognizing the biomolecule. A solution of the biomolecule can then be passed over the surface of the glass slide in a suitable aqueous solution. The biomolecule can selectively bind to the highest affinity inhibitors presented on the surface and signals this event by changing the luminescent properties of the sensitizer. Automated scanning laser confocal fluorescence (Fodor S. et
20 al, Science (1991) 251, 767-773; Fodor S., et al. Nature (1993) 364 555-556; Cho C. et al, Science (1993) 261, 1202-1305; Fodor S., et al. Nature (1993) 364 555-556; Rozsnyai L., Angew. Chem. (1992) 31, 759-761; McGall G. et al, Proc. Natl. Acad. Sci. USA (1996), 93, 13555-13560; McGall G. et al., J. Am. Chem. Soc. (1997) 119, 5081-5090) can systematically test each position on the grid for changes in luminescence when the
25 target biomolecule is present.

Other solid supports suitable for use in the methods of the invention are known to those of skill in the art. A solid support is a matrix in a substantially fixed arrangement. Types of solid supports include, but are not limited to, glass, plastics, polymers, metals,
30 metalloids, ceramics, and/or organic compounds. Solid supports can be planar, flat, or can have substantially different conformations. For example, the sensitizer-linked

substrate can be attached to particles, beads, strands, precipitates, gels, sheets, tubing, spheres, containers, capillaries, pads, slices, films, plates, and/or slides. Magnetic beads or particles (e.g. magnetic latex beads and iron oxide particles) are representative of the solid support to which the sensitizer-linked substrate molecules can be displayed.

5 Magnetic particles are described, (e.g. U.S. Pat. No. 4,672,040) and are commercially available (for example from, PerSeptive Biosystems, Inc. (Framingham, Mass.), Ciba Corning (Medfield, Mass.), Bangs Laboratories (Carmel, Ind.), and BioQuest, Inc. (Atkinson, N.H.)).

10 In addition to screening for potential inhibitors of a P-450 or another enzyme, this approach may also be used to test the binding profile of an uncharacterized P-450 mammalian isozyme, and thereby lend important insight into the structure and functional characteristics of its binding site.

15 **Therapeutic Applications**

The methods of the invention further include therapeutic methods of inhibiting or killing a cell having a target biomolecule on the cell surface. The method of the invention uses sensitizer-linked substrate molecules to specifically target a biomolecule on a selected
20 cell surface and use of the sensitizer to generate an active species, such as the free radical superoxides, to inhibit growth of the cell or to kill the cell. The method applies photodynamic therapies (PDTs) to target biomolecules on selected cells.

As used herein, PDT is largely a method for the treatment of neoplastic and selected
25 nonneoplastic pathogenic diseases. The illumination of a photosensitizer with light of selected wavelength and intensity can induce energy transfer to triplet oxygen which can result in the formation of cytotoxic singlet oxygen. PDT offers the potential for selective ablation of tumor cells and other pathogens. The three PDT requirements are the drug (photosensitizer), light, and oxygen. Thus, PDT is most amenable to the treatment of
30 skin conditions. Sensitizers can be topically applied and have been approved for a variety of dermatological cancers, but drugs with particular affinity for an affected organ

may also be ingested. The Food and Drug Administration has approved PDT for the treatment of advanced esophageal and lung cancers, as well as retinal macular degeneration.

- 5 Two of the greatest challenges in the development of PDT are the need for greater cell discrimination (ideally, exclusive targeting of damaged cells) and the need for modalities that allow greater tissue penetration. The sensitizer-linked substrate molecules of the invention can offer solutions to both problems. The attachment of a photosensitizer to a substrate that specifically recognizes target biomolecules on the surfaces of cancerous
- 10 cells, for example, can offer distinct advantages in selectivity and targeting over current drugs, which rely on nonspecific hydrophobic interactions. The development of sensitizers that absorb at red wavelengths (> 600 nm) and possess high singlet oxygen quantum yields opens possibilities for treatments involving 2-photon excitation. Such modalities allow greater tissue penetration. A.M. Rouhi, "Let There Be Light and Let It Heal," C. & EN. News, Nov. 2, 1998 pp. 22-27; R.A. Hsi, D.I. Rosenthal, E. Glatstein,
- 15 "Photodynamic Therapy in the Treatment of Cancer," Drugs, 57 (5) 725-734; W. Spiller, H. Kliesch, D. Wohrle, S. Hackbarth, B. Roder, G. Schnurpfeil, "Singlet oxygen quantum yields of different photosensitizers in polar solvents and micellar solutions," Journal of Porphyrins and Phthalocyanines, 2 (2) 145-158; K.O. Zahir and A. Haim, "Yields of
- 20 Singlet Oxygen Produced by the Reaction between the Excited-State of Tris(bipyridine)ruthenium(II) and Triplet Dioxygen in Various Solvents," Journal of Photochemistry and Photobiology A-Chemistry, 63 (2) 167-172.

Advantages of the Invention

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- The invention represents an improvement over present technology, for detecting and characterizing biomolecules, in various ways. For example, (a) there are no requirements for radioactive reagents; (b) the methods take advantage of a known recognition element and binding interaction; (c) there is no requirement for chemical or biochemical
- 30 modification of the target biomolecule; (d) there is no covalent modification of the target biomolecule; (e) there is no requirement for mutant forms of the target biomolecules; (f)

the signal or emission generated by the assay is significantly larger and more robust than those typically obtained using previously known biomolecule probe methodologies; (g) a positive luminescence signal is generated by the presence of a candidate modulator, thus facilitating the identification of specific modulatory agents; (h) there are a large variety of luminescent agents that are available (if the modulator decreases affinity, coumarin dyes can be used, if the modulator increases affinity $[\text{Ru}(\text{bpy})_3]^{2+}$ lumophores can be used); (i) through use of different binding elements and linkers, one can adapt the assays of the invention to screen for modulators of numerous biomolecules; (j) one can assay for a target biomolecule in the presence of others; (k) one can assay multiple isoforms of biomolecules in a single reaction; (l) the assay format does not require that the enzyme be immobilized on a solid support during the course of the assay; and (m) each of the formats described is readily amenable for automation and high-throughput screening.

EXAMPLE I

This example describes the optical detection of cytochrome P450 by sensitizer-linked substrates.

Materials and Methods

Synthesis of Sensitizer-linked substrates

All manipulations were conducted under an argon atmosphere using standard Schlenk techniques. Solvents used for synthesis were dried, degassed and distilled according to standard procedures (A. J. Gordon, R. A. Ford, *The Chemist's Companion. A Handbook of Practical Data, Techniques, and References* (John Wiley and Sons, New York, 1972). D. D. Perrin, W. L. F. Armarego, *Purification of Laboratory Chemicals* (Butterworth-Heinemann Ltd., Boston, 3rd Ed., 1988).). Reactions were performed at room temperature unless otherwise stated. All reagents were used as received. NMR spectra were recorded on a General Electric QE300, and later a Varian Mercury 300, generally

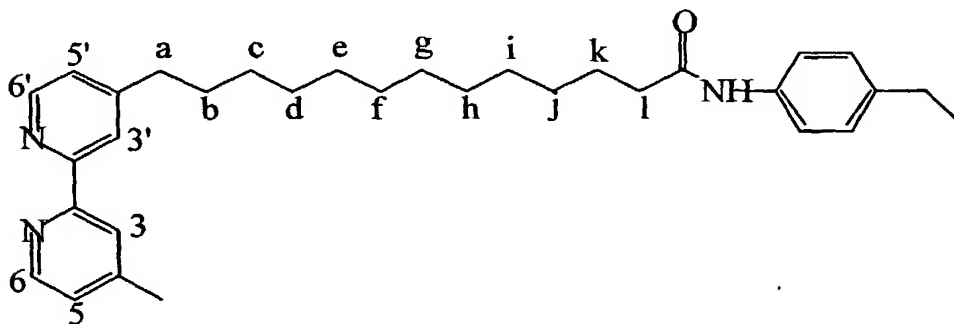
using dry CDCl_3 or CDCl_2 as solvent. ^1H NMR spectral assignments refer to the schematic provided in depicting a typical bpy' ligand used in this study.

General

5

All manipulations were conducted under an argon atmosphere using standard Schlenk techniques. Solvents used for synthesis were dried, degassed and distilled according to standard procedures (A. J. Gordon, R. A. Ford, *The Chemist's Companion. A Handbook of Practical Data, Techniques, and References* (John Wiley and Sons, New York, 1972).

10 D. D. Perrin, W. L. F. Armarego, *Purification of Laboratory Chemicals* (Butterworth-Heinemann Ltd., Boston, 3rd Ed., 1988).). Reactions were performed at room temperature unless otherwise stated. All reagents were used as received. NMR spectra were recorded on a General Electric QE300, and later a Varian *Mercury* 300, generally using dry CDCl_3 or CDCl_2 as solvent. ^1H NMR spectral assignments refer to the
15 schematic provided in depicting a typical bpy' ligand used in this study.



Synthesis of Ru-Substrates and Ru-Ligands

20

Synthesis of bpy-C₉-Ad.

The synthesis of bpy-C₉-Ad is typical of all bpy-C_x-Ad complexes presented. Thionyl chloride (24.50 g, 206 mmol) and 8-bromooctanoic acid (5.46 g, 24.5 mmol) were

combined and refluxed for 1.5 h. Excess SOCl_2 was removed by vacuum to yield a brown liquid that was dissolved in ether (20 mL) and transferred to an addition funnel. The acid chloride was added over 20 min to an ether (20 mL) solution of 2-adamantanamine hydrochloride (11.97 g, 63.8 mmol) and triethylamine (22.50 g, 222 mmol) chilled in an ice bath. The resulting slurry was stirred at 0 °C for 3 h and then overnight at room temperature. The reaction solution was added to water (75 mL) and extracted with ether (75 mL) in a separatory funnel. After washing the organic layer with 0.1 M HCl (3 x 75 mL), water (2 x 75 mL), and saturated brine (2 x 75 mL), the solution was dried over MgSO_4 and solvent removed by rotary evaporation. The off-white solid was used directly without purification for attachment to Me_2bpy .

Diisopropylamine (8.09 g, 79.9 mmol), *n*-butyl lithium (80 mmol in hexanes), and cold THF (25 mL) were combined in a 500 mL Schlenk flask at 0 °C. A cold solution of 4,4'-dimethyl-2,2'-bipyridine (6.41 g, 34.8 mmol) in THF (180 mL) was added by cannula over 15 min, and was stirred for an additional 15 min. The amide was dissolved in THF (120 mL) and cannulated dropwise into the bipyridine, turning the solution from burgundy to black. After 3 h on an ice bath, the reaction was allowed to proceed overnight at room temperature. The reaction solution was transferred to a separatory funnel with water (250 mL) and extracted with ether (150 mL). The organic layer was washed with saturated NaHCO_3 (2 x 125 mL), water (3 x 300 mL), and saturated brine (2 x 200 mL). After drying with MgSO_4 and vacuum, a beige solid was obtained. The product was eluted as the second band by silica gel column chromatography (3:2 ethyl acetate/hexanes). Yield was 3.40 g (30.2% based on 8-bromooctanoic acid) of a pale yellow oil. ^1H NMR (CDCl_3): δ 1-2 (m's), 2.20 (t, CH_2 -amide), 2.42 (s, bpy- CH_3), 2.59 (t, bpy- CH_2), 4.09 (m), 5.79 (m), 7.21 (d, bpy 5 and 5'), 8.23 (s, bpy 3 and 3'), 8.58 (d, bpy 6 and 6').

Synthesis of bpy- C_{11} -Ad.

The synthesis of this compound is similar to that described for bpy- C_9 -Ad. The starting material 2-adamantanamine hydrochloride necessitated the addition of 3.5 equivalents of

triethylamine. ^1H NMR (CDCl_3): δ 1-2 (m's), 2.21 (t, CH_2 -amide), 2.42 (s, bpy- CH_3), 2.60 (t, bpy- CH_2), 4.02 (m), 5.78 (m), 7.19 (d, bpy 5 and 5'), 8.23 (s, bpy 3 and 3'), 8.59 (d, bpy 6 and 6').

5 Synthesis of bpy- C_{10} .

This compound was prepared in a manner analogous to that of bpy- C_{16} . ^1H NMR (CDCl_3): δ 0.82 (t, chain $-\text{CH}_3$), ~1.3 (m, $\text{CH}_2\text{c-i}$), 1.78 (p, CH_2b), 2.55 (s, bpy- CH_3), 2.79 (t, bpy- CH_2), 7.31 (t, bpy 5 and 5'), 8.51 (s, bpy 3 or 3'), 8.59 (s, bpy 3 or 3'), 8.70 (t, bpy 6 and 6').

Synthesis of bpy- C_{16} .

Diisopropylamine (5.79 g, 57.2 mmol), *n*-butyl lithium (57.3 mmol in hexanes) and cold THF (30 mL) were combined in a 500 mL Schlenk flask over an ice bath. A cold solution of 4,4'-dimethyl-2,2'-bipyridine (4.56 g, 24.7 mmol) in 180 mL of THF was added by cannula over 5 min. To this solution was added 1-bromohexadecane (6.29 g, 21.6 mmol) in THF (50 mL). The reaction was stirred on ice for ~3 h, then allowed to warm to room temperature for further stirring overnight. The reaction solution was transferred to a 1 L separatory funnel with water (250 mL) and ether (150 mL). The organic layer was washed with saturated NaHCO_3 (2 x 125 mL), water (2 x 300 mL) and saturated brine (3 x 200 mL). After drying with MgSO_4 , solvent was removed by vacuum and the product purified by silica gel column chromatography using 4:1 hexanes:ethyl acetate as eluent. Yield was 2.40 g (34.4%) of a white solid. ^1H NMR (CDCl_3): δ 0.89 (t, chain $-\text{CH}_3$), ~1.3 (m, $\text{CH}_2\text{c-o}$), 1.75 (p, CH_2b), 2.57 (s, bpy- CH_3), 2.80 (t, bpy- CH_2), 7.33 (t, bpy 5 and 5'), 8.52 (s, bpy 3 or 3'), 8.55 (s, bpy 3 or 3'), 8.67 (t, bpy 6 and 6').

Synthesis of bpy-C₁₁-Br.

This compound was prepared in a manner analogous to that of bpy-C₁₃-Br. ¹H NMR (CDCl₃): δ ~1.7 (m, CH₂c-i), 1.71 (p, CH₂b), 1.79 (p, CH₂j), 2.43 (s, bpy-CH₃), 2.70 (t, bpy-CH₂), 3.40 (t, CH₂-Br), 7.18 (d, bpy 5 and 5'), 8.25 (s, bpy 3 and 3'), 8.59 (t, bpy 6 and 6').

Synthesis of bpy-C₁₃-Br.

Diisopropylamine (0.770 g, 7.61 mmol), *n*-butyl lithium (7.60 mmol in hexanes) and THF (10 mL) were combined in a Schlenk flask at -70 °C. The solution was warmed to 0 °C over 15 min. The solution was again chilled to -70 °C and 1,12-dibromododecane (25.0 g, 7.62 mmol) added as a solid. After warming to 0 °C, stirring continued for ~4 h. The solution was transferred to a separatory funnel to which water (15 mL) and ether (15 mL) were added. The organic layer was washed with saturated NaHCO₃, dried with MgSO₄ and evaporated to a solid under vacuum. Silica gel column chromatography with CHCl₃ as the eluent yielded 1.31 g (40.0%) of a white solid. ¹H NMR (CDCl₃): δ ~1.7 (m, CH₂c-k), 1.75 (p, CH₂b), 1.85 (p, CH₂l), 2.55 (s, bpy-CH₃), 2.76 (t, bpy-CH₂), 3.48 (t, CH₂-Br), 7.26 (d, bpy 5 and 5'), 8.40 (d, bpy 3 and 3'), 8.65 (t, bpy 6 and 6').

Synthesis of bpy-C₁₁-Im.

This compound was prepared by a procedure identical to that of bpy-C₁₃-Im. ¹H NMR (CDCl₃): δ ~1.3 (m, CH₂c-i), 1.72 (m, CH₂b and j), 2.45 (s, bpy-CH₃), 2.72 (t, bpy-CH₂), 3.91 (t, CH₂-imid), 6.82 (s, imid H-5), 7.09 (s, imid H-4), 7.20 (d, bpy 5 and 5'), 7.45 (s, imid H-2), 8.21 (s, bpy 3 and 3'), 8.55 (t, bpy 6 and 6').

Synthesis of bpy-C₁₃-Im.

Imidazole (1.0 g, 15 mmol) and bpy-C₁₃-Br (0.30 g, 0.70 mmol) were combined in a flask with THF (50 mL) and refluxed for 4 days. Solvent was removed under vacuum and the

resulting solid was dissolved in CHCl_3 for washing by saturated NaHCO_3 , water and saturated brine. The product was purified by silica gel column chromatography using ethyl acetate as eluent to yield 0.26 g (90%) of a white solid. ^1H NMR (CDCl_3): δ ~1.3 (m, $\text{CH}_2\text{c-k}$), 1.71 (p, CH_2b), 1.76 (p, CH_2l), 2.42 (s, bpy- CH_3), 2.73 (t, bpy- CH_2), 3.95 (t, CH_2 -imid), 6.90 (s, imid H-5), 7.10 (s, imid H-4), 7.19 (d, bpy 5 and 5'), 7.68 (s, imid H-2), 8.24 (s, bpy 3 and 3'), 8.60 (t, bpy 6 and 6').

Synthesis of bpy-C₇-EB.

10 This compound was prepared by a procedure analogous to that provided for bpy-C₁₁-EB. ^1H NMR (CDCl_3): δ 1.24 (t, ethyl- CH_3), 1.42 (m, CH_2c and d), 1.77 (m, CH_2b and e), 2.39 (t, CH_2 -amide), 2.59 (q, ethyl- CH_2), 2.60 (s, bpy- CH_3), 2.77 (t, bpy- CH_2), 7.11 (d, benzene), 7.29 (d, bpy 5 or 5'), 7.42 (d, bpy 5 or 5'), 7.51 (d, benzene), 8.08 (s, br, amide-H), 8.58 (s, bpy 3 and 3'), 8.69 (t, bpy' 6 and 6').

15

Synthesis of bpy-C₉-EB. This compound was prepared by a procedure analogous to that provided for bpy-C₉-EB. ^1H NMR (CDCl_3): δ 1.19 (t, ethyl- CH_3), 1.40 (m, $\text{CH}_2\text{c-f}$), 1.77 (p, CH_2g), 1.80 (p, CH_2b), 2.42 (t, CH_2 -amide), 2.60 (q, ethyl- CH_2), 2.75 (s, bpy- CH_3), 2.81 (t, bpy- CH_2), 7.11 (d, benzene), 7.50 (d, benzene), 7.59 (m, bpy 5 and 5'), 8.10 (s, br, amide-H), 8.75 (m, bpy 3 and 3'), 9.00 (m, bpy 6 and 6').

20

Synthesis of bpy-C₁₀-EB.

This compound was prepared by a procedure analogous to that provided for bpy-C₁₁-EB. ^1H NMR (CDCl_3): δ 1.26 (t, ethyl- CH_3), 1.36 (m, $\text{CH}_2\text{c-g}$), 1.74 (m, CH_2b and h), 2.34 (t, CH_2 -amide), 2.52 (s, bpy- CH_3), 2.61 (q, ethyl- CH_2), 2.75 (t, bpy- CH_2), 7.15 (d, benzene), 7.26 (m, bpy 5.5'), 7.47 (d, benzene), 8.41 (s, amide-H), 8.45 (m, bpy 3 and 3'), 8.60 (t, bpy 6 and 6').

25

30 Synthesis of bpy-C₁₁-EB.

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The synthesis of bpy-C₁₁-EB is typical for all bpy-C_x-EB compounds presented. Thionyl chloride (19.6 g, 165 mmol) and 9-bromononanoic acid (5.16 g, 20.5 mmol) were combined and refluxed for 2.5 h. Excess SOCl₂ was removed by vacuum to yield a brown solution that was transferred to an addition funnel. The acid chloride was added over 5 min to an ether (20 mL) solution of 4-ethylaniline (6.63 g, 54.7 mmol) chilled on an ice bath. The resulting slurry was stirred on the ice bath for ~ 3 h and then overnight at room temperature. Water (75 mL) and ether (75 mL) were added to the reaction solution in a separatory funnel. After washing of the organic layer with 0.1 M HCl (3 x 75 mL), water (2 x 75 mL) and saturated brine (2 x 75 mL), the solution was dried over MgSO₄ and solvent removed under vacuum. This grey/brown solid amide is used for attachment to 4,4'-dimethyl-2,2'-bipyridine without purification. Yields of the final ligand are highest when the amide is used immediately after preparation.

Diisopropylamine (2.92 g, 28.9 mmol), *n*-butyl lithium (28.8 mmol in hexanes) and cold THF (25 mL) were combined in a 500 mL Schlenk flask chilled over an ice bath. A cold solution of 4,4'-dimethyl-2,2'-bipyridine (2.30 g, 12.5 mmol) in 120 mL of THF was added by cannula over 15 min. To this solution was added the amide in THF (90 mL) by cannula over 15 min. After ~3 h on the ice bath, the reaction was allowed to proceed overnight at room temperature. The reaction solution was transferred to a separatory funnel with water (250 mL) and ether (150 mL). The organic layer was washed with saturated NaHCO₃ (2 x 125 mL), water (3 x 300 mL) and saturated brine (2 x 200 mL). After drying with MgSO₄ and vacuum, a beige solid resulted. The product was purified by silica gel column chromatography using 3:1 hexanes:ethyl acetate for an eluent. Yield was 1.52 g (16.2% based on 9-bromononanoic acid) of a white solid. ¹H NMR (CDCl₃): δ 1.19 (t, ethyl-CH₃), 1.25 (m, CH₂c-h), 1.73 (m, CH₂b and i), 2.28 (t, CH₂-amide), 2.41 (s, bpy-CH₃), 2.60 (q, ethyl-CH₂), 2.71 (t, bpy-CH₂), 7.17 (d, benzene), 7.18 (m, bpy 5.5'), 7.22 (s, amide-H), 7.38 (d, benzene), 8.22 (s, bpy 3 and 3'), 8.56 (t, bpy 6 and 6').

Synthesis of bpy-C₁₂-EB.

This compound was prepared by a procedure analogous to that provided for bpy-C₁₁-EB.

¹H NMR (CDCl₃): δ 1.23 (t, ethyl-CH₃), 1.24 (m, CH₂c-i), 1.73 (m, CH₂b and j), 2.28 (t, CH₂-amide), 2.49 (s, bpy-CH₃), 2.68 (q, ethyl-CH₂), 2.75 (t, bpy-CH₂), 7.22 (d, benzene), 7.30 (m, bpy 5.5'), 7.43 (d, benzene), 7.41 (s, amide-H), 8.29 (s, bpy 3 and 3'), 8.60 (t, bpy 6 and 6').

Synthesis of bpy-C₁₃-EB.

This compound was prepared by a procedure analogous to that provided for bpy-C₁₁-EB.

¹H NMR (CDCl₃): δ 1.20 (t, ethyl-CH₃), 1.26 (m, CH₂c-j), 1.74 (m, CH₂b and k), 2.29 (t, CH₂-amide), 2.43 (s, bpy-CH₃), 2.70 (q, ethyl-CH₂), 2.79 (t, bpy-CH₂), 7.18 (d, benzene), 7.21 (m, bpy 5.5'), 7.40 (s, amide-H), 7.45 (d, benzene), 8.29 (s, bpy 3 and 3'), 8.61 (t, bpy 6 and 6').

Synthesis of [Ru(bpy)₂(bpy-C₉-Ad)]Cl₂.

The synthesis of [Ru(bpy)₂(bpy-C₉-Ad)]Cl₂ is typical of all [Ru(bpy)₂(bpy')]Cl₂ complexes presented here. The ligand bpy-C₉-Ad (505 mg, 1.10 mmol) and *cis*-[Ru(bpy)₂Cl₂] (538.6 mg, 1.04 mmol) were combined with 5:1 water/ethanol (18 mL) and refluxed for 12 h. Solvent was removed under vacuum and the dark red solid was dissolved in water (60 mL). This aqueous solution was combined with a solution of NH₄PF₆ (1.20 g, 7.36 mmol) in water (20 mL) to yield an orange precipitate. The aqueous slurry was extracted with CH₂Cl₂ (75 mL); the organic layer was washed with 1 M HCl (2 x 50 mL), 1 M NaOH (2 x 50 mL), and water (2 x 75 mL) prior to rotary evaporation. The PF₆⁻ salt of this ruthenium complex was purified by silica gel flash chromatography (column dimensions 30 x 4.5 cm) employing an eluent of 3% methanol in CH₂Cl₂. Pure product PF₆⁻ salt was found in elution volumes 550 – 1300 mL. Further product could be obtained by running a second column on the initial 200 – 550 mL. Volumes 550 – 1300 mL were combined and dried by rotary evaporation.

In order to metathesize the ruthenium complex to the Cl^- salt, the purified PF_6^- salt was dissolved in MeOH (10 mL) and loaded onto a CM Sepharose cation exchange column (2 x 13 cm). The column was washed with water (600 mL) and 25 mM NaCl (600 mL).

5 The ruthenium complex was then eluted with 500 mM NaCl (300 mL) and dried by vacuum. The desired $[\text{Ru}(\text{bpy})_2(\text{bpy}-\text{C}_9\text{-Ad})]\text{Cl}_2$ was isolated from the NaCl-containing solid by repeated washings with CH_2Cl_2 , followed by filtering and drying under vacuum. Yield of the dark red solid was 195 mg (20.0%). Yields of this procedure are generally 20–30%, and approach 60% with repeated column chromatography on the crude reaction
10 mixture. ^1H NMR (CD_2Cl_2): δ 0.8–2 (m's), 2.21 (t, CH_2 -amide), 2.65 (s, $\text{bpy}'\text{-CH}_3$), 2.78 (t, $\text{bpy}'\text{-CH}_2$), 3.62 (m), 3.95 (m), 6.32 (m), 7.23 (m), 7.45 (m), 7.70 (m), 8.18 (m), 8.77 (s), 8.80 (s), 9.20 (m). LRMS (electrospray, positive ion) calcd for $\text{C}_{50}\text{H}_{57}\text{N}_7\text{ORu}$ ($\text{M} + \text{H}^+$) m/z 874, found 874. UV-vis [λ ($\Delta\epsilon$), H_2O]: 206 nm (74,200), 244 (26,000), 286 (80,100), 454 (14,500).

15

Synthesis of $[\text{Ru}(\text{bpy})_2(\text{bpy}-\text{C}_{11}\text{-Ad})]\text{Cl}_2$.

This complex was prepared by a procedure similar to that described for $[\text{Ru}(\text{bpy})_2(\text{bpy}-\text{C}_9\text{-Ad})]\text{Cl}_2$. ^1H NMR (CD_2Cl_2): δ 0.8–2 (m's), 2.18 (t, CH_2 -amide), 2.64 (s, $\text{bpy}'\text{-CH}_3$),
20 2.81 (t, $\text{bpy}'\text{-CH}_2$), 3.96 (m), 5.90 (m), 7.25 (m), 7.49 (m), 7.72 (m), 8.19 (m), 8.59 (s), 8.69 (s), 9.95 (m). LRMS (electrospray, positive ion) calcd for $\text{C}_{52}\text{H}_{61}\text{N}_7\text{ORu}$ ($\text{M} - \text{H}^+$) m/z 900, found 900.

Synthesis of $[\text{Ru}(\text{bpy})_2(\text{bpy}-\text{C}_{10})]\text{Cl}_2$.

25

This complex was prepared by a procedure similar to that described for $[\text{Ru}(\text{bpy})_2(\text{bpy}-\text{C}_9\text{-Ad})]\text{Cl}_2$. ^1H NMR (CD_2Cl_2): δ 0.91 (t, chain- CH_3), ~1.3 (m, $\text{CH}_2\text{c-i}$), 1.76 (p, CH_2b),
2.72 (s, $\text{bpy}'\text{-CH}_3$), 2.81 (t, CH_2a), 7.27 (m), 7.51 (m), 7.72 (m), 8.15 (m), 8.74 (s), 8.79 (s), 9.22 (m). LRMS (electrospray, positive ion) calcd for $\text{C}_{41}\text{H}_{46}\text{N}_6\text{Ru}$ ($\text{M} - \text{H}^+$) m/z 723,
30 found 723.

Synthesis of [Ru(bpy)₂(bpy-C₁₆)]Cl₂.

This complex was prepared by a procedure similar to that described for [Ru(bpy)₂(bpy-C₉-Ad)]Cl₂. ¹H NMR (CD₂Cl₂): δ 0.90 (t, chain-CH₃), ~1.3 (m, CH₂c-o), 1.77 (p, CH₂b), 2.69 (s, bpy'-CH₃), 2.80 (t, CH₂a), 7.29 (m), 7.55 (m), 7.76 (m), 8.20 (m), 8.75 (s), 8.80 (s), 9.21 (m). LRMS (electrospray, positive ion) calcd for C₄₇H₅₈N₆Ru (M - H⁺) *m/z* 807, found 807.

10 Synthesis of [Ru(bpy)₂(bpy-C₁₁-Im)]Cl₂.

This complex was prepared by a procedure similar to that described for [Ru(bpy)₂(bpy-C₉-Ad)]Cl₂. ¹H NMR (CD₃OD): δ ~1.3 (m, CH₂c-i), 1.78 (CH₂b and j), 2.65 (s, bpy'-CH₃), 2.76 (t, bpy'-CH₂), 4.01 (t, CH₂-imid), 6.92 (s, imid H-5), 7.12 (s, imid H-4), 7.33 (m), 7.49 (m), 7.68 (m), 7.75 (m), 8.19 (t), 8.62 (s), 8.68 (s), 8.74 (d).

Synthesis of [Ru(bpy)₂(bpy-C₁₃-Im)]Cl₂.

This complex was prepared by a procedure similar to that described for [Ru(bpy)₂(bpy-C₉-Ad)]Cl₂. ¹H NMR (CD₂Cl₂): δ ~1.3 (m, CH₂c-k), 1.72 (CH₂b and l), 2.60 (s, bpy'-CH₃), 2.85 (t, bpy'-CH₂), 3.99 (t, CH₂-imid), 7.04 (m), 7.26 (m), 7.48 (m), 7.71 (m), 8.13 (m), 8.63 (s), 8.70 (s), 9.07 (m). LRMS (electrospray, positive ion) calcd for C₄₇H₅₄N₈Ru (M + H⁺) *m/z* 833, found 833.

25 Synthesis of [Ru(bpy)₂(bpy-C₇-EB)]Cl₂.

This complex was prepared by a procedure similar to that described for [Ru(bpy)₂(bpy-C₉-Ad)]Cl₂. ¹H NMR (CD₂Cl₂): δ 1.05 (t, ethyl-CH₃), 1.25 (m, CH₂c and d), 1.51 (p, CH₂e), 1.70 (p, CH₂b), 2.35 (t, CH₂-amide), 2.40 (q, ethyl-CH₂), 2.52 (s, bpy'-CH₃), 2.80 (t, bpy'-CH₂), 6.82 (d, benzene), 7.20 (m), 7.40 (m), 7.60 (m), 7.73 (d, benzene), 8.00

(m), 9.05 (m), 9.18 (s). LRMS (electrospray, positive ion) calcd for $C_{46}H_{47}N_7ORu$ ($M - H^+$) m/z 814, found 814.

Synthesis of $[Ru(bpy)_2(bpy-C_9-EB)]Cl_2$.

5

This complex was prepared by a procedure similar to that described for $[Ru(bpy)_2(bpy-C_9-Ad)]Cl_2$. 1H NMR (CD_2Cl_2): δ 1.21 (t, ethyl- CH_3), 1.30 (m, CH_2c-f), 1.60 (p, CH_2g), 1.77 (p, CH_2b), 2.45 (t, CH_2 -amide), 2.52 (q, ethyl- CH_2), 2.58 (s, bpy' - CH_3), 2.65 (t, bpy' - CH_2), 7.05 (d, benzene), 7.26 (t), 7.50 (m), 7.70 (m), 7.78 (d, benzene), 8.15 (m), 8.85 (s), 9.00 (t). LRMS (electrospray, positive ion) calcd for $C_{48}H_{51}N_7ORu$ ($M - H^+$) m/z 842, found 842.

10

Synthesis of $[Ru(bpy)_2(bpy-C_{10}-EB)]Cl_2$.

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This complex was prepared by a procedure similar to that described for $[Ru(bpy)_2(bpy-C_9-Ad)]Cl_2$. 1H NMR (CD_2Cl_2): δ 1.13 (t, ethyl- CH_3), 1.21 (m, CH_2c-g), 1.52 (p, CH_2h), 1.68 (p, CH_2b), 2.41 (t, CH_2 -amide), 2.52 (q, ethyl- CH_2), 2.58 (s, bpy' - CH_3), 2.83 (t, bpy' - CH_2), 7.00 (d, benzene), 7.24 (t), ~7.4 (m), 7.67 (m), 7.73 (d, benzene), ~8.1 (m), 8.83 (s), 8.92 (m), 9.05 (m). LRMS (electrospray, positive ion) calcd for $C_{49}H_{53}N_7ORu$ ($M - H^+$) m/z 856, found 856.

20

Synthesis of $[Ru(bpy)_2(bpy-C_{11}-EB)]Cl_2$.

This complex was prepared by a procedure similar to that described for $[Ru(bpy)_2(bpy-C_9-Ad)]Cl_2$. 1H NMR (CD_2Cl_2): δ 1.17 (t, ethyl- CH_3), 1.25 (m, CH_2c-h), 1.61 (p, CH_2i), 1.77 (p, CH_2b), 2.48 (t, CH_2 -amide), 2.59 (q, ethyl- CH_2), 2.63 (s, bpy' - CH_3), 2.90 (t, bpy' - CH_2), 7.06 (d, benzene), 7.29 (m), ~7.5 (m), 7.78 (d, benzene), ~8.1 (m), 8.83 (s), 8.93 (s), ~9.1 (m). LRMS (electrospray, positive ion) calcd for $C_{50}H_{55}N_7ORu$ ($M - H^+$) m/z 870, found 870.

30

Synthesis of [Ru(bpy)₂(bpy-C₁₂-EB)]Cl₂.

This complex was prepared by a procedure similar to that described for [Ru(bpy)₂(bpy-C₉-Ad)]Cl₂. ¹H NMR (CD₂Cl₂): δ 1.22 (t, ethyl-CH₃), 1.32 (m, CH₂c-i), 1.60 (p, CH₂j),
5 1.72 (p, CH₂b), 2.40 (t, CH₂-amide), 2.56 (q, ethyl-CH₂), 2.60 (s, bpy'-CH₃), 2.74 (t, bpy'-CH₂), 7.08 (d), 7.25 (d), ~7.5 (m), 7.70 (m), 8.10 (m), 8.65 (s), 8.72 (s), 8.95 (m), 9.65 (s). LRMS (electrospray, positive ion) calcd for C₅₁H₅₇N₇ORu (M - H⁺) *m/z* 884, found 884.

10 Synthesis of [Ru(bpy)₂(bpy-C₁₃-EB)]Cl₂.

This complex was prepared by a procedure similar to that described for [Ru(bpy)₂(bpy-C₉-Ad)]Cl₂. ¹H NMR (CD₂Cl₂): δ 1.25 (CH₂c-j), 1.28 (t, ethyl-CH₃), 1.61 (p, CH₂k),
1.74 (p, CH₂b), 2.46 (t, CH₂-amide), 2.59 (q, ethyl-CH₂), 2.62 (s, bpy'-CH₃), 2.81 (t, bpy'-
15 CH₂), 7.10 (d), 7.26 (t), ~7.5 (m), 7.73 (m), ~8.1 (m), 8.59 (s), 8.62 (s), ~8.9 (m). LRMS (electrospray, positive ion) calcd for C₅₂H₅₉N₇ORu (M - H⁺) *m/z* 898, found 898.

Synthesis of *para*-methoxy-*N,N*-dimethylaniline.

20 This quencher was prepared according to published procedures (M. Sekiya, M. Tomie, N. J. Leonard, *J. Org. Chem.* 33, 318-322 (1968)). *para*-methoxyaniline (*p*-anisidine, Aldrich, 5.0 g) was placed in a flask with neat methyl iodide (15 mL), fit with a condenser and refluxed for 12 hours under argon. The precipitated iodide salt of the quaternary amine was filtered and dried under high vacuum. Crude yield = 11.62 grams,
25 98% by weight. The aniline salt was put in a flask with 1.5 equivalents of NaOH (2.4 g) and amyl alcohol as solvent (50 mL). Refluxing for 6 hours (~ 152 °C) gave a yellow solution which was filtered on a coarse frit to remove the brown precipitate. The filtrate was washed in a separatory funnel with saturated NaCl solution (100 mL). The top (dark brown) organic layer was dried with MgSO₄, and a short path distillation removed the
30 amyl alcohol (59-79 °C) under high vacuum (~ 1 mm Hg).

Column chromatography was performed in the dark, using 50% EtOAc/50% hexanes as eluent. Three bands emerged; the first band was a fluorescent impurity, the second and third bands were the di- ($r_f \sim 0.7$) and monomethyl aniline ($r_f \sim 0.65$), respectively. Rotary evaporation of the middle fractions gave the product in modest yield (~ 1 gram, 16% based on starting *p*-anisidine). The off-white solid was further purified by sublimation (high vacuum, heated at 30 °C, collected on a small cold finger). Product isolated at this stage was sufficiently pure for reductive quenching purposes. Due to its short shelf-life (stored at 4 °C, under argon, protected from light), the compound was frequently recrystallized from warm water before use. ^1H NMR (CDCl_3): δ 3.30 (s, N-CH₃, 6H), 3.88 (s, O-CH₃, 3H), 7.58 (d, 2H), 7.15 (d, 2H). GC-MS calcd for C₉H₁₃NO ($M - \text{H}^+$) m/z 150, found 150.

Synthesis of 4'-(dimethylamino)-benzo-15-crown-5.

4-aminobenzo-15-crown-5 (Aldrich, 610 mg) and MeI (5 mL) were refluxed for 3 hours under argon while stirring. The solution was filtered to remove most MeI, and the gray powder was dried on a high vacuum line. The solid was put in a 25 mL round bottom flask with Na₂CO₃ (0.00132 mol, 0.140 g) and amyl alcohol (3 mL) and refluxed under argon for five hours. The amyl alcohol was washed twice with water, and dried on a high vacuum line overnight. Column chromatography proved unable to separate the monomethyl and dimethyl products, which appeared to be stoichiometric by NMR.

Thus, the mixture was reacted with 1 equivalent of decanoyl chloride (to generate the amide from the monomethyl impurity and render it separable by column chromatography). Dropwise addition of the acid chloride was performed in chloroform at 0 °C. The reaction ran 6 hours, and was extracted with water. Most of the desired product was in the aqueous phase, and was isolated by rotary evaporation. Purification was achieved by silica gel chromatography (EtOAc, 6% MeOH, 1% NEt₃), and the first few fractions contained the pure dimethyl aniline derivative (colorless oil). Yield = 125 mg, 19% based on starting crown ether. ^1H NMR (CDCl_3): δ 2.85 (s, N-CH₃, 6 H), 3.67 (m, -CH₂CH₂-, 8H), 3.81 (m, -CH₂, 2H), 3.84 (m, -CH₂, 2H), 4.01 (m, -CH₂, 2H), 4.08

(m, -CH₂, 2H), 6.24 (pair of doublets, 1 H), 6.80 (d, 1 H), 6.36 (d, 1 H), 6.80 (d, 1 H). ESI (electrospray, positive ion) calcd for C₁₆H₂₅NO₅ (M - H⁺) *m/z* 312, found 312. Also found, 334 (+ Na⁺) and 350 (+ K⁺). The 15-crown-5 ether derivative prefers sodium, but binds both cations in the gas phase.

5

Synthesis of 2-adamantylacetamide.

The hygroscopic white solid 2-adamantylamine was prepared by dissolving 2-adamantylamine•HCl (Aldrich) in H₂O/NaOH, extracting with methylene chloride, drying with MgSO₄, filtering, and rotary evaporating. This reagent (500 mg) was dissolved in methylene chloride (20 mL), put on ice, and acetic anhydride (~ 5 equiv.) added dropwise. The reaction was left to run overnight, and worked up by addition of sodium bicarbonate solution, and extraction with MeCl₂. The solution was washed twice with H₂O and dried with MgSO₄. The white crystalline product looked clean by TLC (50% CH₂Cl₂/EtOAc, imaged with paraanisaldehyde, *r_f* ~ 0.25) and NMR without further purification. ¹H NMR (CDCl₃): δ 1.62-1.90 (m's, 15 H), 4.05 (br's, -CH₃), 5.82 (br's, N-H, amide).

15

Synthesis of 4-(N-imidazole)-2,2',3,3',4',5,5',6,6'-fluorobiphenyl.

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Perfluorobiphenyl (133.6 mg, 0.4 mmol), imidazole (27.2 mg, 0.4 mmol), and K₂CO₃ (55.3 mg, 0.4 mmol) were put in a round bottom flask with freshly distilled DMSO, and the reaction was run under argon for 24 hours at 30 °C. Work up involved adding 25 mL H₂O and extracting three times with 25 mL MeCl₂. The organic layer was dried over MgSO₄, filtered, and rotovapped. TLC (EtOAc) showed two spots (monoimidazole, *r_f* = 0.75; diimidazole, *r_f* = 0.5; consumed starting perfluorobiphenyl, *r_f* = 1). The mixture was purified on silica gel using 50% EtOAc/hexanes as the eluent. Yield = 75 mg (49%) of the desired monoimidazole product with nearly an equal amount of the bisimidazole compound. ¹H NMR (CD₂Cl₂): δ 7.20 (br, 1H), 7.23 (m, 1H), 7.78 (br, 1H). ¹⁹F NMR (CD₂Cl₂): δ -129 (m, 2F), -130 (m, 2F), -139 (m, 2F), -141.5 (m, 2F), -151.7 (m, 1F). ESI (electrospray, positive ion) calcd for C₁₅H₃N₂F₉ (M - H⁺) *m/z* 383, found 383.

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Synthesis of 4,4'-bis(N,N'-imidazole)-2,2',3,3',5,5',6,6'-fluorobiphenyl.

Isolated in the procedure above, this compound was synthesized in nearly quantitative yields by using three equivalents of imidazole and K_2CO_3 . The product was purified on silica gel using 70% EtOAc/hexanes as eluent. 1H NMR (CD_2Cl_2): δ 7.32 (br, 1H), 7.37 (m, 1H), 7.87 (br, 1H). ^{19}F NMR (CD_2Cl_2): δ -136.9 (m, 4F), -148 (d, 4F). ESI (electrospray, positive ion) calcd for $C_{18}H_6N_4F_8(M - H^+)$ m/z 431, found 431.

10 Synthesis of 4,4',5,5'-tetramethyl-2,2'-bipyridine.

This bipyridyl ligand (Me_4bpy) was synthesized following published procedures (G. A. Mines, et al., *J. Am. Chem. Soc.* **118**, 1961-1965 (1996)). The brown liquid lutidine (3,4-dimethylpyridine, Aldrich, 477 g, 4.45 mol) and Pd/C (10% Pd on carbon, Aldrich, ~ 40 g) were combined in a 2-liter flask with a reflux condenser, refluxed and stirred for 8 days. While still hot, the black solution was filtered on celite and cooled on ice. The beige crystals were collected with a Büchner funnel, washed with ether, and recrystallized with 1:1 $CHCl_3$ /toluene. The off-white crystals crashed out of solution in the freezer, and were collected by filtration. Yield = 18.9 g, 4.0%. 1H NMR ($CDCl_3$): δ 2.31 (s, - CH_3 , 6H), 2.37 (s, - CH_3 , 6H), 8.20 (s, 2H), 8.40 (s, 2H).

Synthesis of $[Ru(Me_4bpy)_2Cl_2] \cdot 2H_2O$.

This was synthesized by a modification of published procedures (S. Gould, T. R. O'Toole, T. J. Meyer, *J. Am. Chem. Soc.* **112**, 9490-9496 (1990)). $RuCl_3 \cdot 3H_2O$ (Aldrich, 927 mg, 3.56 mmol), Me_4bpy (1.50 g, 7.08 mmol), LiCl (2.23 g, 52.6 mmol), and hydroquinone (EM, 1.96 g, 17.8 mmol) were dissolved in anhydrous dimethoxyethane (100 mL) and distilled methanol (50 mL). The solution was purged with argon for 20 minutes and refluxed under argon for 24 hours. Water (225 mL) was added to the cooled solution, and the brownish purple solid was collected on a medium frit and washed thoroughly with H_2O . The product was dissolved in $MeCl_2$ (450 mL) and washed with

water (3 x 450 mL) until the aqueous layer was colorless. The organic layer was dried with MgSO_4 and rotary evaporated. The dark purple powder was reprecipitated from minimal MeCl_2 with ether, collected by filtration, and dried under high vacuum; yield = 600 mg, 28%.

5

Synthesis of $[\text{Ru}(\text{tmbpy})_2(4\text{-hydroxymethyl-4'-methylbipyridine})](\text{PF}_6)_2$.

$\text{Ru}(\text{tmbpy})_2\text{Cl}_2$ (100 mg, 0.168 mmol) and 4-hydroxymethyl-4'-methylbipyridine (73.9 mg, 0.369 mmol, synthesized exactly according to published procedures (14)) were put in a round bottom flask with H_2O (5 mL) and EtOH (2 mL) and refluxed under argon for 3 hours. The ethanol was removed by rotary evaporation, and orange crystals were obtained by adding a concentrated solution of $(\text{NH}_4)\text{PF}_6$, filtering on a frit and drying on an aspirator. Purification was performed on an 8" alumina column eluting with 2:1 toluene/acetonitrile. A dark nonfluorescent band eluted first, followed by the major orange band (product); brown and red junk stuck to the column. Yield = 40 mg, 25%.

15

A general methodology was developed to synthesize Ru-probes in good yields and with minimal effort. The first chromatography step generally gave 30% yields of the pure bpy' ligand, based on the starting bromoamide. Non-fluorescing silica TLC plates were used for all bpy ligand syntheses, since bpy coordinates the metal in the fluorescing plates, causing the spot to streak. The TLC plates were stained with a ferric salt solution, which turned the bpy spots red and made imaging easy, quick, and non-toxic. The second chromatography step was tried on ion exchange as well as alumina media before settling on silica gel as the best support.

20

The ruthenation step generally yielded 60% pure Ru-substrate, while for Ru-ligands this final step yielded only ~ 30%. Elute the Ru-compounds with nitrate in the solvent--this minimizes streaking and isolates the product as the water soluble nitrate salt (obviating the need for metathesis). Metathesis was not always time consuming, however; it was possible to dissolve the more hydrophobic $[\text{Ru-substrate}](\text{PF}_6)_2$ salts in dry acetone, and metathesize directly with tetrabutylammonium chloride, avoiding ion exchange

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chromatography completely. Unfortunately, due to the high solubility of many Ru-substrate chloride salts in both organic and aqueous solutions, this was not always possible. Cation exchange chromatography often served as a final purification, as well as metathesis step.

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The synthesis of *p*-MDMA was quite straightforward; the purification, however, was not. Unfortunately, separation of the mono and di-methyl products proved difficult. One tip worth following would be to react the mixture with decanoyl chloride, as was done to synthesize 4'-(dimethylamino)-benzo-15-crown-5. The conversion of the monomethyl side product to the decyl amide should make purification much easier.

10

Finally, $\text{Ru}(\text{Me}_4\text{bpy})_2(\text{Cl})_2 \cdot 2\text{H}_2\text{O}$ is a useful precursor for many high driving force excited-state ET reactions. The redox potential generally decreases 20 mV/methyl group, making $[\text{Ru}(\text{Me}_4\text{bpy})_2(\text{dmbpy})]^{2+} \sim 200$ mV more negative than $\text{Ru}(\text{bpy})_3$. Several different variants were synthesized to tune the driving force (i.e., $\text{Ru}(\text{dmbpy})_2\text{Cl}_2$) or to make the complex reactive with surface cysteines (i.e., $\text{Ru}(\text{tmbpy})_2(4\text{-bromomethyl-4'-methylbipyridine})[\text{PF}_6]_2$) (L. Geren, S. Hahm, B. Durham, F. Millet, *Biochemistry* **30**, 9450-9457 (1991)), but it is left to the reader to explore these other avenues.

15

20 Protein Isolation

P450_{cam} Expression/Crystallization Conditions. *P. putida* cytochrome P450_{cam} (residues 1-414) containing the mutation Cys334Ala (Quickchange mutagenesis, Stratagene) was overexpressed in *E. coli* TB19 cells from plasmid pUS200 (Unger, B. P., et al. (1986) *J. Biol. Chem.* 261, 1158-1163) and purified in the presence of camphor as previously described (Nickerson, D., et al. (1998) *Acta Crystallogr.* D54, 470-472). P450:Ru-C₉-Ad seed crystals of space group P2₁2₁2₁ (cell dimensions 65.4 x 74.5 x 91.7 Å³, one molecule/asymmetric unit, Matthews coefficient (V_M) = 2.4, solvent content = 49%) nucleated overnight (4 °C, vapor diffusion) from protein separated from camphor and complexed with stoichiometric Ru-C₉-Ad. Hanging drops contained an equal volume mixture of reservoir and 430 μM P450:Ru-C₉-Ad in 20 mM HEPES pH 7.5, 100 mM KCl, and 1 mM dithiothreitol. The reservoir contained 100 mM NaOAc pH 4.9, 200 mM

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NH₄OAc pH 7.0, and 9-11% polyethylene glycol (PEG) MW 8000 (W/V) (final pH ~6.0). Diffraction quality crystals (0.15 x 0.15 x 0.5 mm³) were grown over 24-48 hours by moving seed crystals into sitting drops of reduced PEG concentrations (5-7%).

5 *Structure Determination.*

An initial molecular replacement solution (correlation coefficient = 0.53 and $R_{\text{cryst}} = \Sigma ||F_{\text{obs}}| - |F_{\text{calc}}|| / \Sigma |F_{\text{obs}}| = 43.4\%$, for 15.0 to 3.5 Å resolution data) was found by AMoRe (Navaza, J. (1994) *Acta Crystallogr. A* **50**, 157-163) with a probe derived from the
10 structure of camphor-bound P450_{cam}, PDB code: 2cpp (Poulos, T. L., et al. (1987) *J. Mol. Biol.* **195**, 687-700), using diffraction data collected from P450:Ru-C₉-Ad crystals (1.55 Å resolution, overall $R_{\text{sym}} = \Sigma \Sigma_j |I_j - \langle I \rangle| / \Sigma \Sigma_j I_j = 4.8\%$, overall signal-to-noise ratio = $I/\sigma I = 37.4$, redundancy = 6.5, 99.2% complete). Diffraction data were collected at 100 K on
15 Beam-line 7-1 (1.08 Å) of the Stanford Synchrotron Research Laboratory (SSRL) and processed with DENZO (Otwinowski, Z. & Minor, W. (1997) *Meth. Enzymol.* **276**, 307-326). Substantial changes in the regions of P450 distal to the heme were modeled to omit electron density maps with XFIT (McRee, D. E. (1992) *J. Mol. Graphics* **10**, 44). Ru-C₉-Ad was positioned into the remaining difference density. The structure was refined by
20 torsion-angle molecular dynamics and positional refinement with CNS (Brünger, A. T., et al. (1998) *Acta Crystallogr. D* **54**, 905-921) amidst model rebuilding, water molecule placement, and resolution extension to 1.55 Å. Following an overall anisotropic thermal factor correction, bulk-solvent correction, and individual thermal factor refinement, grouped occupancy refinement of Ru-C₉-Ad and those residues in multiple conformations produced the final model (4019 scatterers, 1 Ru-C₉-Ad as a superposition of the two (Δ
25 and Λ) {Ru(bpy)₃}²⁺ enantiomers, 23 residues in multiple conformations, 427 water molecules, and 5 acetate molecules; $R_{\text{cryst}} = 21.6\%$, $R_{\text{free}} = 22.6\%$ for 8% of the reflections removed at random, no σ cutoff). The adamantyl moiety of Ru-C₉-Ad is well ordered, but static and/or dynamic disorder increases up the methylene chain toward the sensitizer, where only one of the three bpy ligands is well resolved. The ruthenium atom
30 position was confirmed by the largest peak in the initial $F_{\text{obs}} - F_{\text{calc}}$ electron density map (4 σ) and also by a peak in the Bijvoet difference Fourier map (calculated with

coefficients $|F^+| - |F^-|$ and phases $\phi_{\text{model}} - \pi/2$), which identified all sulfur and metal atoms in the model. The final model has excellent stereochemistry (root mean square deviation from ideal bond lengths $< 0.009 \text{ \AA}$ and ideal bond angles $< 1.3^\circ$) with 90.3% of all residues in the most favored regions of ϕ/ψ space, as defined by PROCHECK (Laskowski, R. A., et al. (1993) *J. Appl. Crystallogr.* **26**, 283-291). No residues fall in disallowed regions. Larger refined thermal factors for Ru-C₉-Ad ($\langle B \rangle = 48.2 \text{ \AA}^2$) compared to the overall model ($\langle B \rangle = 28.0 \text{ \AA}^2$, $\langle B \rangle_{\text{mainchain}} = 19.4 \text{ \AA}^2$, $\langle B \rangle_{\text{sidechain}} = 20.4 \text{ \AA}^2$) reflect the mobility and conformational heterogeneity of the bound $\{\text{Ru}(\text{bpy})_3\}^{2+}$. The ribbon representation (Figure 1) was generated using Molscript (Kraulis, P. J. (1991) *J. Appl. Cryst.* **24**, 946-950) and Raster3D (Merritt, E. A. & Bacon, D. J. (1997) *Methods Enzymol.* **277**, 505-524). The methylene linker occupies a large channel from the enzyme surface to the heme. A hydrogen bond connects the Ru-substrate amide carbonyl (red atom) to Tyr 96 (orange). The adamantyl moiety (center) resides at the P450 active site above the heme (orange) in the same position as the natural adamantane substrate (magenta), shown in superposition from the 4cpp crystal structure (Raag, R. & Poulos, T. L. (1991) *Biochemistry* **30**, 2674-2684). Although both Δ and Λ $\{\text{Ru}(\text{bpy})_3\}^{2+}$ enantiomers are present in the complex, only Λ is shown.

Energy Transfer Measurements

Solution experiments were performed under an argon atmosphere with P450 and Ru-substrate in 100 mM KCl and 20 mM KPhos buffer, pH 7.4. Single crystal experiments were conducted aerobically. Samples were excited with XeCl excimer-pumped dye laser pulses (25 ns, 480 nm, 1-2 mJ/pulse). The emission decay traces were fit to the biexponential function, $y = c_0 + c_1 e^{-(k_{\text{en}} + k_0)t} + c_2 e^{-k_0 t}$. The ratio $c_1:(c_1 + c_2)$ was used to calculate dissociation constants. Donor-acceptor spectral overlap gives a Förster distance (Ru-Fe distance at which half the emission is quenched by energy transfer) (Förster, T. (1965) in *Modern Quantum Chemistry*, ed. Sinanoglu, O. (Academic Press, New York), Vol. 3, pp. 93-137) of $R_0 = 26.2 \text{ \AA}$ for the ferriheme enzyme and $R_0 = 27.6 \text{ \AA}$ for the carbonmonoxy species. Ru-Fe distances, r , were calculated using the equation, $k_{\text{en}} = k_0(R_0/r)^6$.

Results and Discussion

Ru-substrates (Figure 2) were modeled into the substrate-free P450 crystal structure (Poulos, T. L., et al. (1986) *Biochemistry* **25**, 5314-5322) to position ethyl benzene (EB) and adamantane (Ad) at the active site and $\{\text{Ru}(\text{bpy})_3\}^{2+}$ at the protein surface. Ru-C_n-EB and Ru-C_n-Ad were constructed by the covalent attachment of EB and Ad to variable length methylene chains $[(\text{CH}_2)_{7-13}]$ terminating in the photosensitizer (Wilker, J. J., et al. (1999) *Angew. Chem. Int. Ed.* **38**, 90-92). An amide functionality was incorporated into the Ru-substrates to permit hydrogen bonding, as occurs between Tyr 96 and camphor (Poulos, T. L., et al. (1987) *J. Mol. Biol.* **195**, 687-700). To generate Ru-ligands that could bind the heme iron (Dawson, J. H., et al. (1982) *J. Biol. Chem.* **257**, 3606-3617), imidazole was linked to alkyl-tethered $\{\text{Ru}(\text{bpy})_3\}^{2+}$ (Ru-C_n-Im). Ru-EB/Ad compounds, as well as Ru-Im, have been shown to bind P450 with high affinity (Wilker, J. J., et al. (1999) *Angew. Chem. Int. Ed.* **38**, 90-92). One of these complexes, P450:Ru-C₉-Ad, was crystallized and structurally characterized to 1.55 Å (Figure 1). The Ru-substrate binds as predicted, with the Ad moiety mimicking substrate (Raag, R. & Poulos, T. L. (1991) *Biochemistry* **30**, 2674-2684), a hydrogen bond between Tyr 96 and the amide functionality, and $\{\text{Ru}(\text{bpy})_3\}^{2+}$ at the mouth of a large channel that has opened to accommodate the sensitizer.

Binding of the Ru-C_n-EB/Ad/Im compounds to the P450 target was detected by decreases in Ru²⁺ excited-state (Ru^{2+*}) lifetimes (Wilker, J. J., et al. (1999) *Angew. Chem. Int. Ed.* **38**, 90-92). $[\text{Ru-substrate}]^{2+*}$ emission decay is normally monophasic ($k_0 = 2.1 \times 10^6 \text{ s}^{-1}$), but becomes biphasic with a dominant fast component ($k_{\text{en}} = 0.5\text{-}1.4 \times 10^7 \text{ s}^{-1}$; $k_0 = 2.1 \times 10^6 \text{ s}^{-1}$) in the presence of P450 (Figure 3). A secondary (< 10%) slower phase ($k_0 = 4.8 \times 10^6 \text{ s}^{-1}$) also was observed, suggesting that a small percentage of the Ru-substrate may remain unbound in the crystal. Faster $\{\text{Ru}(\text{bpy})_3\}^{2+*}$ emission decay, k_{en} , in the crystal relative to solution most likely reflects small conformational differences in P450 between the two phases. Faster decay of the intrinsic $\{\text{Ru}(\text{bpy})_3\}^{2+*}$ emission, k_0 , in the crystal is attributable to quenching by oxygen. Thus, upon addition of enzyme, the Ru-substrate or

Ru-ligand partitions between a “bound” state, in which Ru^{2+} is quenched, and a “free” state, in which it is not. Photoexcitation of a P450:Ru-C₉-Ad single crystal yields a predominantly monophasic luminescence decay (Figure 3) that is strongly quenched by the protein ($k_{\text{en}} = 1.1 \times 10^7 \text{ s}^{-1}$), thereby confirming that the fast decay component, k_{en} , is attributable to P450:Ru-substrate complex formation.

Competitive binding between Ru-substrates and camphor at the active site is indicated by the ability of the natural substrate ($K_{\text{D}} \sim 1 \text{ } \mu\text{M}$) to diminish the fraction of bound $[\text{Ru-substrate}]^{2+}$ decaying at the faster rate, k_{en} . At the titration end-point, camphor completely displaces the Ru-substrate from P450, as shown by monophasic Ru^{2+} emission decay kinetics ($k_0 = 2.1 \times 10^6 \text{ s}^{-1}$). Analysis of Ru-C₁₁-Ad emission quenching by P450 yields a dissociation constant ($K_{\text{D}} = 0.8 \text{ } \mu\text{M}$) in excellent agreement with Ru-C₁₁-Ad/camphor competitive binding assays monitored by UV/Vis spectroscopy ($K_{\text{D}} = 0.7 \text{ } \mu\text{M}$). Association of Ru-substrates and Ru-ligands with P450 is sufficiently strong to allow detection of the enzyme at submicromolar concentrations (Figure 4). The emission decay profile of Ru-C₉-Ad (2.5 μM) in 50 mM sodium phosphate buffer, pH 7, was monophasic ($k_0 = 2.1 \times 10^6 \text{ s}^{-1}$) in the presence of six heme proteins (yeast cytochrome *c*, horse skeletal muscle myoglobin, bovine lipase cytochrome *b*₅, bovine liver catalase, recombinant yeast cytochrome *c* peroxidase, and horseradish peroxidase), each at 5 μM . The finding that the addition of 500 nM P450_{cam} to this mixture yielded biphasic Ru^{2+} kinetics ($\sim 10\% k_{\text{en}}$, $\sim 90\% k_0$) demonstrates the feasibility of detecting specific target biomolecules in complex media (Figure 5).

Specificity of Ru-substrates for P450 is controlled largely by interactions of the substrate moiety with the active site. Particularly noteworthy is the fact that $\{\text{Ru}(\text{bpy})_3\}^{2+}$ is a sensitive reporter of binding even for substrates that do not shift the heme absorption spectrum by displacing ligated water (Wilker, J. J., et al. (1999) *Angew. Chem. Int. Ed.* **38**, 90-92). Dissociation constants for Ru-C_n-EB compounds are the first presented for derivatives of ethyl benzene. The chain-length dependence of binding in the Ru-C_n-EB series ($K_{\text{D}} = 0.7\text{-}6.5 \text{ } \mu\text{M}$ for $n = 7\text{-}13$) demonstrates that detection of P450 by Ru-substrates may be fine-tuned by modification of the linker component. In the case of Im-

terminated tethers, however, Ru-C₁₁-Im has low affinity for P450, whereas Ru-C₁₃-Im binds the enzyme tightly (Figure 4). Apparently, the shorter linker does not allow the Im to extend far enough into the protein to ligate the heme iron.

- 5 Förster (dipole-dipole) energy transfer (FET) dominates the quenching in P450:Ru-substrate complexes. Evidence that electron transfer does not contribute significantly to this quenching is the finding that ferriheme reduction by {Ru(bpy)₃}⁺ is ~10³ times slower than k_{en} (Wilker, J. J., et al. (1999) *Angew. Chem. Int. Ed.* **38**, 90-92). Spectral overlap of {Ru(bpy)₃}²⁺ emission with the absorption of Fe(CO)²⁺ P450 is greater than
10 with the ferriheme enzyme, suggesting that FET should be more efficient in the carbonyl complex (where both oxidation and reduction of the heme-CO complex are energetically disfavored). Not only is the decay of Ru²⁺ in P450 Fe(CO)²⁺:Ru-C₁₁-EB 1.5 times faster ($k_{\text{en}} = 1.6 \times 10^7 \text{ s}^{-1}$), the calculated Ru-Fe distances differ by only 0.4 Å for the two heme oxidation states (Förster, T. (1959) *Discussions Faraday Soc.* **27**, 7-17; Galley, W. C. &
15 Stryer, L. (1969) *Biochemistry* **8**, 1831-1833).

The Ru-Fe distance found in the P450:Ru-C₉-Ad crystal (21 Å) is in excellent agreement with the Förster analysis of energy-transfer kinetics for this complex in solution. Similar Ru-Fe distances were calculated for the various Ru-substrates, suggesting a common
20 mode of Ru-substrate binding at the P450 active site. The shallow Ru-Fe distance dependence on chain length in the Ru-C_n-EB series confirms that {Ru(bpy)₃}²⁺ always binds at the protein surface. The shortest ethyl benzene derivatives, Ru-C₇-EB and Ru-C₉-EB, report the minimum length of the substrate access channel to be 19.5 Å. These data also indicate that the region occupied by the methylene linker represents the most
25 likely path followed by natural substrates to access the P450 active center. The swath cut by Ru-substrates in P450 is a channel of considerable breadth (3-8 Å) and depth (~20 Å).

This example demonstrates the novel method of the invention for sensing specific biomolecules that involves tethering a photosensitizer to a substrate molecule with high
30 affinity for an active site of a target biomolecule. Analysis of Ru/heme FET kinetics revealed the dimensions and conformational flexibility of the access channel, and probed

the mechanism of substrate binding. This approach can be broadly expanded through a combinatorial approach to designing substrate moieties that target P450s as well as other enzymes, modifying sensitizers to produce desired signals, and optimizing linkages to fine-tune specificity or probe target conformations. Replacement of $\{\text{Ru}(\text{bpy})_3\}^{2+}$ with osmium polypyridyl complexes (Kober, E. M., et al. (1985) *Inorg. Chem.* **24** 2755-2763) would tune the emission further towards the near infrared, thereby improving tissue penetration and optical detection over the background of scattered light from cellular components. The ability of sensitizer-linked substrates to detect proteins and perform photochemical oxidation and reduction reactions at specific enzyme active sites (Wilker, J. J., et al. (1999) *Angew. Chem. Int. Ed.* **38**, 90-92) opens new avenues for intervention in metabolic processes.

EXAMPLE II

15 *This example describes enantiomeric discrimination of Ru-substrates by cytochrome*
P450_{cam}

It was found that substrates and ligands attached via an alkyl chain to the inorganic photosensitizer $[\text{Ru}(\text{bpy})_3]^{2+}$ (where bpy is 2,2'-bipyridine) bind P450 reversibly [Wilker, J.J. et al. *Angew. Chem. Int. Ed.* (1999), 38, 90-92] with high affinity ($K_D \sim 1 \mu\text{M}$) and specificity [as described in Example I *infra*, Dmochowski, I.J., et al. *Proc. Natl. Acad. Sci. USA* (1999), 96, 12987-12990]. The substrate $[\text{Ru}-\text{C}_9\text{-Ad}]\text{Cl}_2$ (Figure 6) was recently crystallized with P450 and the X-ray structure determined to 1.55 Å (PDB code, 1qmq; Figure 7) [as described in Example I *infra*, Dmochowski, I.J., et al. *Proc. Natl. Acad. Sci. USA* (1999), 96, 12987-12990]. The adamantyl moiety resides in the heme pocket, much like the substrate adamantane. Electron density from the ruthenium and bipyridyl ligands appears in multiple positions near the substrate channel, thereby indicating either considerable mobility of the $\{\text{Ru}(\text{bpy})_3\}^{2+}$ moiety or the existence of stable enzyme-Ru conjugates that could correspond to specific interactions of Λ and Δ enantiomers with the protein surface. High thermal factors for the $\{\text{Ru}(\text{bpy})_3\}^{2+}$ moiety in the crystal structure prevented unambiguous assignment of either isomer. The inherent

chirality of both P450 and $\{\text{Ru}(\text{bpy})_3\}^{2+}$ raises the possibility that hydrophobic interactions with aromatic residues at the channel entrance favor the binding of one isomer relative to the other. This potential enantioselectivity was probed by resolving the Λ and Δ $[\text{Ru-C}_9\text{-Ad}]\text{Cl}_2$ isomers and comparing their affinities for P450.

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Chromatographic techniques using SP Sephadex C-25 with chiral eluents have been developed for the separation of many enantiomeric ruthenium polypyridyl complexes [Fletcher, N.C.; Keene, F.R.; *J. Chem. Soc., Dalton Trans.* (1999), 5 683-689; Fletcher, N.C. et al., *J. Chem. Soc. Dalton* (1998), 1 133-138; Rutherford, T.J. et al., *Eur. J. Inorg. Chem.* (1998), 11 1677-1688; Rutherford, T.J. et al., *Inorg. Chem.* (1995), 34 3857-3858]. The Sephadex ion exchange matrix itself is chiral, since it is made of dextran, a 3-dimensional network of cross-linked D-glucose units. Interestingly, the ability of dextran to achieve chiral resolutions of d^6 (Re^{I} , Ru^{II} , Os^{II} , Co^{III} , Rh^{III}) polypyridyl compounds is greatly enhanced by the addition of tartrate salts [Yoshikawa, Y.; Yamasaki, K. *Coord. Chem. Rev.* (1979), 28 205-229]. X-ray structures of these metal complexes crystallized with aromatic tartrate counterions (i.e., (+)-O,O'-di-4-toluoyl-D-tartrate) show well-ordered stacking interactions between the ditoluoyl and phenanthroline groups [Yoshikawa, Y.; Yamasaki, K. *Coord. Chem. Rev.* (1979), 28 205-229]. Well-defined structures incorporating a variety of organic salts also have been observed in solution by ^1H NMR [Fletcher, N.C.; Keene, F.R.; *J. Chem. Soc., Dalton Trans.* (1999), 5 683-689]. Aromatic stacking has been implicated as a major factor in the mechanism of stereoisomer separation with these eluents [Rutherford, T.J. et al., *Eur. J. Inorg. Chem.* (1998), 11 1677-1688], and provides a mechanism for chiral discrimination by the enzyme. In this example, sodium (-)-O,O'-dibenzoyl-L-tartrate was chosen for the isolation of the (+)- $[\text{Ru-C}_9\text{-Ad}]\text{Cl}_2$ isomers because it most efficiently resolves the parent compound, $[\text{Ru}(\text{bpy})_2(\text{Me}_2\text{bpy})]\text{Cl}_2$ (Me_2bpy is 4,4'-dimethyl-2,2'-bipyridine).

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Time-resolved luminescence measurements precisely quantify the binding of Ru-substrates to P450 [Wilker, J.J. et al. *Angew. Chem. Int. Ed.* (1999), 38, 90-92; as described in Example I *infra*, Dmochowski, I.J., et al. *Proc. Natl. Acad. Sci. USA* (1999), 96, 12987-12990]. Laser excitation of the Ru-protein solutions yields biphasic

luminescence kinetics. The faster quenching process ($k = 4-14 \times 10^6 \text{ s}^{-1}$, depending on substrate and chain length) has been identified as Förster energy transfer from Ru^{2+*} to the heme [as described in Example I *infra*, Dmochowski, I.J., et al. *Proc. Natl. Acad. Sci. USA* (1999), 96, 12987-12990]. The slower luminescence decay process ($\tau \sim 500 \text{ ns}$) is the same as that of Ru^{2+*} in deoxygenated solution. Thus, dissociation constants can be calculated from the quenched fraction of $[\text{Ru-substrate}]^{2+*}$ luminescence. Traditional P450 substrate-binding assays rely on monitoring the low-to-high-spin shift (417 392 nm) associated with water loss from a ferric-aquo heme. Time-resolved emission profiles much more reliably assess the affinity of substrates (e.g., $(+)-[\text{Ru-C}_9\text{-Ad}]\text{Cl}_2$) that displace little water from the channel and only slightly perturb the spin state of the heme.

Materials and Methods

Protein Preparation

Cytochrome P450_{cam} was overexpressed in *E. coli* TBV cells from plasmid pUS200 [Unger, B.P. et al., *J. Biol. Chem.* (1986), 261 1158-1163] and purified in the presence of camphor according to standard procedures [Nickerson, D. et al., *Acta Crystallogr.* (1998), D54 470-472].

Synthesis of $[\text{Ru-C}_9\text{-Ad}]\text{Cl}_2$

General Procedures

All manipulations were conducted under an argon atmosphere using standard Schlenk techniques. Solvents used for synthesis were dried, degassed and distilled according to standard procedures [Perrin, D.D.; Armarego W.L.F. *Purification of Laboratory Chemicals*, 3rd ed.; Butterworth-Heinemann Ltd., Boston, 1988; A.J. Gordon, R.A. Ford *The Chemist's Companion. A Handbook of Practical Data, Techniques, and References*; John Wiley and Sons, New York, 1972]. Reactions were performed at room temperature unless otherwise stated. NMR spectra were recorded on a General Electric QE300.

Synthesis of bpy-C₉-Ad

Thionyl chloride (24.50 g, 206 mmol) and 8-bromooctanoic acid (5.46 g, 24.5 mmol) were combined and refluxed for 1.5 h. Excess SOCl₂ was removed by vacuum to yield a brown liquid that was dissolved in ether (20 mL) and transferred to an addition funnel. The acid chloride was added over 20 min to an ether (20 mL) solution of 2-adamantanamine hydrochloride (11.97 g, 63.8 mmol) and triethylamine (22.50 g, 222 mmol) chilled in an ice bath. The resulting slurry was stirred at 0 °C for 3 h and then overnight at room temperature. The reaction solution was added to water (75 mL) and extracted with ether (75 mL) in a separatory funnel. After washing the organic layer with 0.1 M HCl (3 x 75 mL), water (2 x 75 mL), and saturated brine (2 x 75 mL), the solution was dried over MgSO₄ and solvent removed by rotary evaporation. The off-white solid was used directly without purification for attachment to Me₂bpy.

Diisopropylamine (8.09 g, 79.9 mmol), *n*-butyl lithium (80 mmol in hexanes), and cold THF (25 mL) were combined in a 500 mL Schlenk flask at 0 °C. A cold solution of 4,4'-dimethyl-2,2'-bipyridine (6.41 g, 34.8 mmol) in THF (180 mL) was added by cannula over 15 min, and was stirred for an additional 15 min. The amide was dissolved in THF (120 mL) and cannulated dropwise into the bipyridine, turning the solution from burgundy to black. After 3 h on an ice bath, the reaction was allowed to proceed overnight at room temperature. The reaction solution was transferred to a separatory funnel with water (250 mL) and extracted with ether (150 mL). The organic layer was washed with saturated NaHCO₃ (2 x 125 mL), water (3 x 300 mL), and saturated brine (2 x 200 mL). After drying with MgSO₄ and vacuum, a beige solid was obtained. The product was eluted as the second band by silica gel column chromatography (3:2 ethyl acetate/hexanes). Yield was 3.40 g (30.2% based on 8-bromooctanoic acid) of a pale yellow oil. ¹H NMR (CDCl₃): δ 1-2 (m's), 2.20 (t, CH₂-amide), 2.42 (s, bpy-CH₃), 2.59 (t, bpy-CH₂), 4.09 (m), 5.79 (m), 7.21 (d, bpy 5 and 5'), 8.23 (s, bpy 3 and 3'), 8.58 (d, bpy 6 and 6').

[Ru(bpy)₂(bpy-C₉-Ad)]Cl₂. The ligand bpy-C₉-Ad (505 mg, 1.10 mmol) and *cis*-[Ru(bpy)₂Cl₂] (538.6 mg, 1.04 mmol) were combined with 5:1 water/ethanol (18 mL) and refluxed for 12 h. Solvent was removed under vacuum and the dark red solid was dissolved in water (60 mL). This aqueous solution was combined with a solution of
5 NH₄PF₆ (1.20 g, 7.36 mmol) in water (20 mL) to yield an orange precipitate. The aqueous slurry was extracted with CH₂Cl₂ (75 mL); the organic layer was washed with 1 M HCl (2 x 50 mL), 1 M NaOH (2 x 50 mL), and water (2 x 75 mL) prior to rotary evaporation. The PF₆⁻ salt of this ruthenium complex was purified by silica gel flash chromatography (column dimensions 30 x 4.5 cm) employing an eluent of 3% methanol
10 in CH₂Cl₂. Pure product PF₆⁻ salt was found in elution volumes 550 – 1300 mL. Further product could be obtained by running a second column on the initial 200 – 550 mL. Volumes 550 – 1300 mL were combined and dried by rotary evaporation. In order to metathesize the ruthenium complex to the Cl⁻ salt, the purified PF₆⁻ salt was dissolved in MeOH (10 mL) and loaded onto a CM Sepharose cation exchange column (2 x 13 cm).
15 The column was washed with water (600 mL) and 25 mM NaCl (600 mL). The ruthenium complex was then eluted with 500 mM NaCl (300 mL) and dried by vacuum. The desired [Ru(bpy)₂(bpy-C₉-Ad)]Cl₂ was isolated from the NaCl-containing solid by repeated washings with CH₂Cl₂, followed by filtering and drying under vacuum. Yield of the dark red solid was 195 mg (20.0%). Yields of this procedure are generally 20 - 30%,
20 and approached 60% with repeated column chromatography on the crude reaction mixture. ¹H NMR (CD₂Cl₂): δ 0.8-2 (m's), 2.21 (t, CH₂-amide), 2.65 (s, bpy'-CH₃), 2.78 (t, bpy'-CH₂), 3.62 (m), 3.95 (m), 6.32 (m), 7.23 (m), 7.45 (m), 7.70 (m), 8.18 (m), 8.77 (s), 8.80 (s), 9.20 (m). LRMS (electrospray, positive ion) calcd for C₅₀H₅₇N₇ORu (M + H⁺) *m/z* 874, found 874. UV-vis [λ (Δε, H₂O)]: 206 nm (74,200), 244 (26,000), 286
25 (80,100), 454 (14,500).

Chiral Resolution of (±)-[Ru-C₉-Ad]Cl₂

Circular dichroism (CD) spectra were measured on samples dissolved in acetonitrile (50-
30 100 μM) using an Aviv Model 62A DS spectropolarimeter. Chiral separation was achieved by cation exchange chromatography (SP Sephadex C-25, Fluka) using 50 mM

sodium (–)-O,O'-dibenzoyl-L-tartrate as the eluent. The aqueous tartrate solution was prepared by neutralization of the acid with two equivalents of NaOH, followed by filtration to remove insoluble impurities. Racemic [Ru-C₉-Ad]Cl₂ (4 mg) was loaded onto a column (dimensions 120 x 3.5 cm) covered with aluminum foil to eliminate the possibility of photoracemization. Eluent flow was regulated (~ 1 mL/min) with a peristaltic pump. The resolution of two bands occurred after traversing an effective column length (ECL) of 2 meters. Upon separation, the Sephadex was expelled from the column with air, and the first and second bands were collected and soaked in acetonitrile to remove Ru from the dextran. The red solutions were rotary evaporated at room temperature, redissolved in water, and metathesized by ion exchange to their chloride salts. Band 1 (the first eluted fraction) had a negative rotation and was assigned the Δ absolute configuration based on the CD characteristics of similar complexes [Rutherford, T.J. et al., *Eur. J. Inorg. Chem.* (1998), 11 1677-1688]. CD [λ (Δε), CH₃CN]: Δ(–): 227 nm (+26), 240 (+23), 260 (–10), 278 (+134), 294 (+307), 325 (+18), 365 (+11), 424 (+19), 476 (–15); Λ(+): 227 (–27), 240 (–23), 260 (+7), 278 (–126), 294 (+281), 325 (–17), 365 (–11), 424 (–19), 476 (+13).

K₁ Determination

A Hewlett Packard 8452A spectrophotometer was used to collect UV-vis data. Buffer conditions were 50 mM potassium phosphate, 100 mM potassium chloride, pH 7.4 for all protein solutions (~ 5 μM P450). UV-vis titrations were performed at 20 °C with stirring (500 rpm) using a Hewlett Packard 89090A stirrer/temperature controller. Λ and Δ-[Ru-C₉-Ad]Cl₂ displace little water from the ferric-aquo heme and binding results in only 30% conversion to the high-spin species, due, presumably, to the abundance of water in Ru-bound (open) structure. Thus, affinities were determined by the ability of these complexes to inhibit the low- to high-spin transition produced by camphor. Concentrated ethanolic stock solutions of camphor titrated in small aliquots (0.5–1.0 μL) into the protein solutions gave the desired range of camphor concentrations (250 nM–2 mM). The concentration of ethanol never exceeded 1% of the total volume. Apparent dissociation constants of camphor, K_S, were spectroscopically determined at three

concentrations (0–20 μM , 99% bound) of both Ru-C₉-Ad isomers. K_S was calculated by fitting the data to $1/\Delta A$ vs. $1/[S]$, the slope of which yields $K_S/([E]\Delta\epsilon)$ from the relationship $1/\Delta A = ((K_S/[S]) + 1)/([E]\Delta\epsilon)$, where ΔA is the absorbance change from the initial value, $[S]$ is the concentration of camphor, $[E]$ is the concentration of P450, and $\Delta\epsilon$ is the difference in molar absorptivity between [Ru-C₉-Ad]- and camphor-bound P450. Absorbance changes were recorded at 392 and 416 nm. Values of K_I , the equilibrium constant between Ru-bound and camphor-bound P450, were determined for both isomers by plotting K_S against the Ru-C₉-Ad concentration. The dissociation constants, K_D , of (+)-[Ru-C₉-Ad]Cl₂ were calculated based on a single-substrate binding model (Figure 7). By definition, $K_D = K_{\text{cam}}/K_I$, where K_{cam} is the dissociation constant of camphor (in the absence of Ru-substrate), and K_I is the equilibrium constant between camphor- and ruthenium-bound P450, spectroscopically determined by measuring the dissociation constant of camphor at multiple Ru concentrations. Luminescence experiments measure K_D directly.

K_D Determination

Emission experiments were conducted under similar conditions (20 °C, buffered solutions, 5 μM in both Ru-substrate and protein). Samples were prepared in a 1-cm-pathlength quartz cuvette with a long neck fitted with a 24/40 joint and a threaded compression seal. The samples (1.5 mL) were deoxygenated by repeated cycles of vacuum followed by argon back-filling. Bubbling of the samples was avoided to minimize protein denaturation. UV-vis spectra were measured routinely before and after each luminescence measurement to verify that the protein samples had not degraded. Nanosecond emission kinetics were fit to the sum of two exponentials ($I(t) = c_0 + c_1\exp(-k_1t) + c_2\exp(-k_2t)$) using an in-house nonlinear least-squares fitting program. Dissociation constants for both isomers were determined using the ratio of the coefficients for the fast and slow phases, $c_1/(c_1+c_2)$.

Time-resolved Emission

The excitation source for all experiments was a tunable (220-2000 nm) optical parametric oscillator (Spectra Physics, MOPO) pumped by a frequency-tripled Q-switched Nd:YAG laser (Spectra Physics, 355 nm, 350 mJ/pulse, 8-ns FWHM). The OPO output power was attenuated by passage through a polarizer; laser shots with energies differing by more than 10% from the mean value (laser pulses detected by a photodiode and selected by a discriminator, Phillips Scientific Model 6930) were rejected. Deoxygenated Ru-protein samples were excited at 470 nm, typically 2 mJ/pulse at the sample. Emission was collected 180° to the incident excitation with reflective optics (f/10), sent through a long-pass filter ($\lambda > 600$ nm), and focused onto the entrance slit of an ISA double 0.1 meter monochromator. Luminescence was detected by a Hamamatsu photomultiplier tube (R928); the output signal passed through a high-speed (100 MHz) current to voltage amplifier, digitized (Sony/Tektronix digitizer, Model RTD710A), and recorded on a personal computer. Instrument response was 10 ns (FWHM). Emission kinetics data are averages of at least 250 laser shots.

Results

Resolution of racemic $[\text{Ru-C}_9\text{-Ad}]^{2+}$ was accomplished by cation-exchange chromatography using a chiral eluent, sodium (-)-O,O'-dibenzoyl-L-tartrate. The CD spectra of the Λ -(+) and Δ -(-)- $[\text{Ru-C}_9\text{-Ad}]\text{Cl}_2$ isomers are shown in Figure 9. In both cases, enantiomeric excess is $> 90\%$ based on the similarity of their extinction coefficients at every wavelength ($< 10\%$ deviation), as well as their similarity to published values for $(\pm)\text{-}[\text{Ru}(\text{bpy})_3]\text{Cl}_2$ [Rutherford, T.J. et al., *Eur. J. Inorg. Chem.* (1998), 11 1677-1688]. Initial efforts to purify $[\text{Ru-C}_9\text{-Ad}]\text{Cl}_2$ in larger quantities (40 mg) and with more concentrated eluent (150 mM) were unsuccessful.

Figure 10 shows a standard low- to high-spin conversion involving the titration of camphor into the P450 active site in the presence of Λ and Δ - $[\text{Ru-C}_9\text{-Ad}]$ isomers. The dissociation constant for camphor alone (K_{cam}) was found to be $3.0 \pm 0.2 \mu\text{M}$ under the

experimental conditions, in good agreement with the literature value [Mueller, E.J. et al. (Ed.): *Twenty-five years of P450_{cam} research, Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd ed., Plenum Press, New York 1995, pp. 83-124]. The steric bulk of Ru-substrates appears to preclude co-occupation of the active site with camphor, an observation supported by the P450:Ru-C₉-Ad crystal structure in which the adamantyl moiety binds above the heme and hydrogen bonds to Tyr 96 much like camphor [Dmochowski, I.J., et al. *Proc. Natl. Acad. Sci. USA* (1999), 96, 12987-12990]. UV-vis absorption measurements of Ru-C₉-Ad displacement show a preference for the Λ isomer (K_D (Λ) = 200 ± 50 nM; K_D (Δ) = 300 ± 50 nM). It was found empirically that displacement of camphor (100 μ M camphor, 4.67 μ M P450, 99% bound) by Ru-substrates yields dissociation constants with higher precision. The apparent dissociation constant, K_D = 240 ± 20 nM, of racemic Ru-C₉-Ad determined by this Ru-titration method (Figure 11) is in excellent agreement with the predicted value (K_D = 248 nM).

Time-resolved luminescence measurements also distinguish the binding of Λ and Δ -[Ru-C₉-Ad] isomers to P450 (Figure 12). The monophasic emission decay ($k = 2.0 \times 10^6$ s⁻¹) of Ru-C₉-Ad alone in solution is nearly identical with that of the slower phases of the two solutions containing P450. This provides strong evidence that binding can be modeled as a two-state equilibrium, and in the "free" state the Ru-substrates are completely dissociated from the protein. Virtually the same quenching rate constants ($k = 4.5 \times 10^6$ s⁻¹) from the Λ and Δ "bound" states indicate comparable Ru-Fe distances for the two isomers. The proportion of the decay (Λ , $87.5 \pm 0.5\%$; Δ , $82.5 \pm 0.5\%$; 5 μ M P450, 5 μ M Ru) that is attributable to this faster (quenched) phase is clearly greater for Λ -[Ru-C₉-Ad]. The K_D values for the enantiomers determined by time-resolved emission (Λ , 90 ± 20 ; Δ , 190 ± 20 nM) are in good agreement with the K_D for racemic Ru-C₉-Ad (150 ± 30 nM).

In order to test whether the single-substrate binding model (Figure 7) accurately describes camphor displacement of Ru-C₉-Ad from P450, a series of UV-vis absorption and emission experiments were performed in parallel. Figure 13 reveals that the amounts of

unbound Ru^{2+} (by luminescence) and low-spin P450 (by UV-vis) track closely during camphor titration into the 1:1 P450:Ru-C₉-Ad complex. Luminescence data (samples at ambient conditions, monitored at 620 nm; kinetics fit to biphasic decay give fraction Ru^{2+} quenched by P450) were collected subsequent to each UV-vis measurement. The
5 spin state of the heme was calculated from changes in absorbance at 416 nm. Luminescence measurements report persistent Ru^{2+} binding at high camphor concentrations. However, persistent quenching of Ru^{2+} at high camphor concentrations suggests that a small fraction (%quenched-%low spin $\sim 5\%$) of Ru-C₉-Ad binds cooperatively in the P450 channel, presumably above the camphor binding site. Energy-
10 transfer kinetics do not change during the titration, indicating that $\{\text{Ru}(\text{bpy})_3\}^{2+}$ remains at the surface ($\sim 20 \text{ \AA}$ from the heme) in this ternary complex.

Confirmation that Λ -[Ru-C₉-Ad] binds P450 with roughly twice the affinity of the Δ isomer comes from an experiment in which buffered P450 (20 μM) and (+)-[Ru-C₉-
15 Ad]Cl₂ (40 μM) were centrifuged together through a size-selective membrane (Centricon, YM-10). CD measurement showed that the Ru-substrate effluent (23 μM , in good agreement with the dissociation constant) was enantiomerically enriched by 15% with the Δ (more weakly bound) isomer. In the absence of P450, no enantiomeric enhancement was found to occur during filtration. Thus, of the 17 μM Ru-C₉-Ad remaining bound to
20 the enzyme, 10.2 μM corresponded to Λ and 6.8 μM to the Δ isomer. The ratio of the concentrations of bound isomers ($\Lambda/\Delta \sim 1.5$) is in reasonable agreement with the corresponding K_D ratio ($\Delta/\Lambda \sim 2$).

Discussion

25

Sodium (-)-O,O'-dibenzoyl-L-tartrate proved much less efficient at resolving (+)-[Ru-C₉-Ad]Cl₂ (ECL = 200 cm) than reported for the model compound $[\text{Ru}(\text{bpy})_2(\text{Me}_2\text{bpy})]\text{Cl}_2$ (ECL = 70 cm) [Rutherford, T.J. et al., *Eur. J. Inorg. Chem.* (1998), 11 1677-1688], presumably due to interference from the long alkyl substituent. Chiral ruthenium
30 polypyridyl compounds have been synthesized directly by starting with one of the enantiomers of $[\text{Ru}(\text{bpy})_2(\text{CO})_2]^{2+}$ [Rutherford, T.J. et al., *Eur. J. Inorg. Chem.* (1998), 11

1677-1688]. Addition of the third bipyridyl ligand occurs with stereoretention if the temperature, solvent, and ligand concentration are carefully controlled [Fletcher, N.C. et al., *J. Chem. Soc. Dalton* (1998), 1 133-138]. This method would seem preferable to chiral separations of functionalized $[\text{Ru}(\text{bpy})_2(\text{bpy}')]\text{Cl}_2$ compounds, which required 2-3 weeks for purification of milligram quantities of material.

That $K_D(\text{energy transfer}) < K_D(\text{UV-vis})$ reflects subtle, method-dependent differences in the quantification of Ru-substrate binding. Nonspecific binding of Ru-C₉-Ad to the enzyme will affect the Ru^{2+} emission decay profile, owing to energy transfer to the heme, but will not perturb the UV-vis spectrum if camphor is in place at the active site. Interestingly, when the Ru-C₉-Ad concentration exceeds that of P450, the luminescence results begin to deviate from predictions based on a single-substrate binding model. Energy-transfer experiments reveal that when $[\text{Ru}] \gg [\text{P450}]$, at least four equivalents of Ru-C₉-Ad associate with the enzyme (5 μM P450, 50 μM Ru; 20 μM Ru quenched at $\sim 20 \text{ \AA}$ from the heme). In fact, the P450 interior is greatly expanded in this open form (Figure 7) and should permit orientations of Ru-C₉-Ad different from that found in the crystallized complex.

An electrostatic map of the protein surface indicates that the entrance to the substrate channel is neutral, favoring hydrophobic rather than electrostatic interactions in recruiting $\{\text{Ru}(\text{bpy})_3\}^{2+}$ to this region, especially at high ionic strengths. Evidence of the dominance of hydrophobic interactions is the finding that bipyridyl-substituted adamantane itself, before ruthenation, strongly binds P450. It also is of interest that hydrophobic interactions appear to play a role in certain stereoselective bimolecular electron-transfer reactions between metalloproteins and inorganic complexes [Sakaki, S. et al, *Inorg. Chem.* (1989) 28, 4061-4063; Sakaki, S. et al., *J. Chem. Soc. Dalton Trans.* (1991) 4, 1143-1148; Pladziewicz, J.R. et al., *Inorg. Chem.* (1993) 32, 2525-2533].

Experiments with other Ru-linked moieties [Wilker, J.J. et al. *Angew. Chem. Int. Ed.* (1999), 38, 90-92; Dmochowski, I.J., et al. *Proc. Natl. Acad. Sci. USA* (1999), 96, 12987-12990] indicate that the terminal group moderately influences the overall affinity of the

Ru-substrate for P450. (Ru-adamantane compounds bind with 3-fold higher affinity than Ru-(ethyl benzene) analogs, and 9-fold higher affinity than unsubstituted Ru-alkyl chains). A comparable effect (K_D increases 9-fold) is observed when short linkers connecting the ethyl benzene to the photosensitizer prohibit optimal positioning of the substrate within the active-site pocket [Dmochowski, I.J., et al. *Proc. Natl. Acad. Sci. USA* (1999), 96, 12987-12990]. Sufficient chain length is especially critical for imidazole-terminated compounds, where the ability to bind the iron is requisite for association. The modest 2-fold discrimination of Λ and Δ -[Ru-C₉-Ad] provides strong evidence that interactions near the protein surface are of lesser importance than the shape complementarity and hydrophobicity of the Ru-substrate in binding to the enzyme.

Enantiospecific binding indicates that noncovalent interactions over 10 Å from the active site impact substrate selection even when the channel is open, as must occur during entrance and egress of natural substrates. Similar long-range secondary interactions also influence the binding of benzenesulfonamide ligands to carbonic anhydrase [Boriak, P.A. et al., *J. Med. Chem.* (1995) 38, 2286-2291]. Based on the P450:Ru-C₉-Ad crystal structure [Dmochowski, I.J., et al. *Proc. Natl. Acad. Sci. USA* (1999), 96, 12987-12990], which confirms the ability of P450 to accommodate large substrates, and identifies hydrophobic interactions of the bipyridyl groups with Phe 193 and Tyr 29 (Figure 7), it can be inferred that aromatic stacking plays an important role in chiral discrimination. Aromatic residues at the mouth of the P450 channel have been implicated previously in the recognition of hydrophobic substrates [Raag, R. et al., *Biochemistry* (1993) 32, 4571-4578].

Sub micromolar affinities, protein specificity, reversible binding, and synthetic versatility make sensitizer-linked substrates ideal for probing P450 active sites. Employing UV-vis and time resolved luminescence measurements, it was found that P450 has a 2-fold preference for Λ -[Ru-C₉-Ad]Cl₂. Emission experiments, especially with highly luminescent {Ru(bpy)₃}²⁺ complexes, are particularly sensitive and convenient for measuring substrate binding. It is well known that the chirality and shape of substrate pocket promote enantio- and regioselective P450 catalysis. It was demonstrated in this

example that long-range interactions with a pendant metal complex also affect substrate binding at the active site of the enzyme.

EXAMPLE III

5

This example provides data for sensitizer-linked substrate molecules as viable substrates for cytochrome P450_{cam}

[Ru-C₉-Ad]Cl₂, despite opening the P450 cavity, is a viable substrate, as shown by this
10 example using electrospray mass spectroscopy assay. The rate and efficiency of [Ru-C₉-
Ad]Cl₂ hydroxylation is compared to the untethered analog, 2-adamantanyl acetamide
(Figure 14). Resonance Raman spectroscopy of Fe²⁺-CO substrate complexes has been
shown previously to be a sensitive reporter of the heme environment (O.
Bangcharoenpaurpong, et al., *J. Chem. Phys.* **87**, 4273-4284 (1987); C. Jung, et al.,
15 *Biochemistry* **31**, 12855-12862 (1992); T. Uno, et al., *J. Biol. Chem.* **260**, 2023-2026
(1985); A. V. Wells, et al., *Biochemistry* **31**, 4384-4393 (1992)), and solution
measurements comparing the binding of Ru-C₉-Ad to adamantane agree with the crystal
structure and substrate turnover results. Experiments attempting P450-mediated Ru-C₉-
Ad hydroxylation with steady-state photolysis suggested that turnover may be occurring
20 in very low yields. In addition, a ternary complex involving P450, camphor, and Ru-C₁₀
(Figure 14) has been characterized by time-resolved emission measurements (while (1)
competes with camphor for the P450 active site, (2) is able to share the pocket with
camphor. 2-adamantylacetamide (2) is analagous to compound (1), without the Ru-
tether; (2) induces a full low to high spin conversion at the heme). By comparing
25 camphor hydroxylation rates in the ternary complex to natural camphor catalysis, the
enzymatic activity of this previously uncharacterized, but potentially biologically relevant
open conformation of the enzyme, was probed.

Materials and Methods

General

- 5 [Ru-C₉-Ad]Cl₂ (1), 2-adamantylacetamide (2), and [Ru-C₁₀]Cl₂ (3) were synthesized as described in Example I. P450, PdR, and Pd were expressed and purified according to literature procedures (J. A. Peterson, et al., *J. Biol. Chem.* **265**, 6066-6073 (1989); P. W. Roome, et al., *J. Biol. Chem.* **258**, 2593-2598 (1983); C. A. Tyson, et al., *J. Biol. Chem.* **247**, 5777-5784 (1972); M. J. Hintz, et al., *J. Biol. Chem.* **257**, 14324-14332 (1982)).
- 10 NADH and adamantane were purchased from Sigma Chemical Co. (St. Louis, Mo) and used without further purification. A miniature oxygen electrode was purchased from Microelectrodes, Inc. (Microelectrodes, Inc., Bedford, NH) and the voltage output calibrated with solutions containing 0%, 21% (ambient), and 100% dioxygen.
- 15 UV-vis titrations were performed using a Hewlett Packard 8452A spectrophotometer and a Hewlett Packard 89090A (Hewlett Packard, Palo Alto, CA) stirrer/temperature controller, at 20 °C with stirring (500 rpm). Time-resolved emission experiments and data analysis with *Kinfit*, *Decon*, and *MATLAB* were performed as described previously (Example II), with the exception that the P450:Ru-C₁₀:camphor ternary complexes were
- 20 not degassed during the titration or subsequent laser experiments.

Resonance Raman Spectroscopy on P450 Fe²⁺-CO Substrate Complexes.

- Samples (200 µL, 100 µM P450 in standard KPi/KCl buffer, substrate concentration = 1
- 25 mM) were prepared in glass NMR tubes fitted with a rubber septum. The solutions were gently bubbled with CO for several minutes before adding a spatula tip of dithionite. Formation of the Fe²⁺-CO complex was verified by UV-vis (λ_{max} = 446 nm). Samples were excited at 441 nm with a HeCd laser (Liconix, Sunnyvale, CA, model 424ONB, 40 mW), and the Raman scatter was focused using longitudinal and transverse collection
- 30 optics onto a double spectrometer (SPEX 1403, 0.85 m) interfaced to a PC via the SPEX MSD2 module. The signal was collected using a PMT (Photomultiplier tube)

(Hamamatsu R955, Hamamatsu City, Japan) powered by Pacific Precision Instruments (1100 V). The sample control unit, photon counter, amplifier/discriminator, and buffered interface were all from EG&G Instruments (Oak Ridge, TN).

5 *Hydroxylation of [Ru-C₉-Ad]Cl₂ with NADH/PdR/Pd/P450.*

A 4 mL solution (20 mM potassium phosphate buffer, 100 mM KCl, pH = 7.4) was prepared containing 1 μ M P450, 1 μ M PdR, 10 μ M Pd, 20 μ M [Ru-C₉-Ad]Cl₂, and 200 μ M NADH ($\epsilon = 6.22 \text{ M}^{-1}\text{cm}^{-1}$ @ 340 nm). The consumption of NADH was monitored at
10 340 nm by UV-vis spectroscopy. Once >95% of the NADH was consumed, the reaction was quenched by the addition of 40 μ L of a 1 M ethanolic camphor solution (ratio camphor/[Ru-C₉-Ad]Cl₂ ~ 500) to displace [Ru-C₉-Ad]Cl₂ from P450 and rapidly consume any remaining NADH. This solution was concentrated by centrifugation
15 saturated phosphate buffer was added to the protein and centrifuged to remove any remaining Ru. The flow-through, containing hydroxylated [Ru-C₉-Ad]Cl₂, camphor, and buffer was rotary evaporated to dryness. Two cycles of acetonitrile (5 mL) addition, decanting, and rotary evaporation were performed to separate the buffer salts from the ruthenated species. The ruthenium concentration was quantified by UV-vis ($\epsilon_{456} =$
20 $14,500 \text{ M}^{-1}\text{cm}^{-1}$). [Ru-C₉-Ad-OH]Cl₂ was diluted to 10 μ M in acetonitrile, and its purity confirmed by electrospray mass spectroscopy (Figure 15).

Attempted Light-activated Hydroxylation of [Ru-C₉-Ad].

25 Steady-state visible irradiation of a 4 mL solution (20 mM potassium phosphate buffer, 100 mM KCl, pH = 7.4) containing 10 μ M P450, 10 μ M [Ru-C₉-Ad]Cl₂, and 100 μ M catalase was performed for two hours. The Ru-complex was extracted as described above and analyzed by ESI.

30 *Electrospray Mass Spectroscopy.*

Samples ($\sim 10 \mu\text{M}$) contained in a 500 μL Hamilton syringe were injected at a rate of 5 $\mu\text{L}/\text{min}$ into the LcQ (Finnigan Mat); typical runs required less than 100 μL per sample, and data sets were averages of 50 scans.

5 *Calibration of ESI (Electrospray Ionization).*

A 10 μM stock solution of $[\text{Ru-C}_9\text{-Ad}]\text{Cl}_2$ in acetonitrile was combined with 10 μM $[\text{Ru-C}_9\text{-Ad-OH}]\text{Cl}_2$ in varying proportions (4:1 to 1:4). Each solution was injected three different times, with intermediary blank runs of pure acetonitrile. Relative peak
10 intensities were determined using analysis software, which deconvolved the spectra to assign one singly charged peak to each compound. A calibration graph (Figure 16) was generated from this data which showed that the ionization efficiency of Ru-C₉-Ad and Ru-C₉-Ad-OH were identical (slope = 0.96) within experimental error. This information was crucial in quantifying hydroxylated product yields relative to starting material in the
15 analysis of subsequent turnover experiments.

Monitoring O₂/NADH Consumption.

The oxygen electrode was calibrated at 20 °C (linear response with $[\text{O}_2]$, 0-100% O_2), and
20 connected to a LeCroy oscilloscope interfaced to a PC using freely available LeCroy software (LeCroy, Chestnut Ridge, NY). The O_2 electrode (3 mm diameter with Teflon casing) was pushed through a septum, which formed an airtight seal with the mouth of a standard 1 cm pathlength cuvette. The quality of the seal was tested by filling the cuvette with deoxygenated buffer and monitoring oxygen concentration; insignificant leakage
25 was observed during 1 hour. All UV-vis experiments were conducted with stirred samples at 20 °C. Kinetics experiments were run in kinetics mode (Biosym) with 1 mL samples containing 1 μM PdR, 10 μM Pd, 1 μM P450, and either 50 μM $[\text{Ru-C}_9\text{-Ad}]\text{Cl}_2$ or 1 mM adamantane. Experiments were initiated upon addition of 200 μM NADH, and absorbance at 340 nm was measured simultaneously with O_2 consumption.

30

Enzyme Turnover of Camphor.

A 4 mL solution (20 mM potassium phosphate buffer, 100 mM KCl, pH = 7.4) was prepared containing 1 μ M P450, 1 μ M PdR, 10 μ M Pd, 400 μ M camphor, and 200 μ M NADH ($\epsilon = 6.22 \text{ M}^{-1}\text{cm}^{-1}$ at 340 nm). The consumption of NADH was monitored at 340 nm by UV-vis spectroscopy and O₂ consumption monitored with the microelectrode. Once all of the NADH was consumed, a known amount of 3-*endo*-bromocamphor was added, and the mixture was extract three times with an equal volume of methylene chloride. The organic extract was concentrated by evaporation and analyzed by GC.

10 GC-MS.

Samples were analyzed by a VG 7070E mass spectrometer in line with an HP 5700 gas chromatograph equipped with a 30-m HP-1 capillary column (0.25 mm inner diameter, film thickness 0.25 μ m) and interfaced with a PC. The organic extract was loaded onto the column at 40 °C, and eluted with a standard program (40 °C – 150 °C, 40 °C/min; 150 °C – 250 °C, 15 °C/min).

Results

20 *Resonance Raman Studies of P450 Fe²⁺-CO:Substrate Complexes*

As shown in Figure 17 and as was reported previously (C. Jung, et al., *Biochemistry* 31, 12855-12862 (1992)), the CO-stretching mode in the P450-carbon monoxide complex is quite sensitive to the nature of the substrate. This indicates a direct interaction between the bound CO and the substrate molecule which agrees with IR absorption work of O'Keefe et al. (D. H. O'Keefe, et al., *Biochemistry* 17, 5845-5852 (1978)). Camphor-bound Fe²⁺-CO P450 has $\nu_{\text{CO}} \sim 1940 \text{ cm}^{-1}$ and camphor-free has two bands at $\sim 1942 \text{ cm}^{-1}$ and 1963 cm^{-1} which have been assigned to a bent and upright geometry, respectively. The Fe-(CO) stretching frequency differs by 4 cm^{-1} between Ru-C₁₁-Ad, Ru-C₉-Ad, and adamantane, suggesting that these substrates bind with slightly increasing proximity to the heme (Figure 18). The fact that these stretching frequencies (472-476 cm^{-1}) are

approximately midway between those found for substrate-free and camphor-bound suggests that CO is bound mostly upright in solution. The similarity between the binding modes of Ru-Ad compounds and adamantane in solution agrees with the X-ray structure determination.

5

Kinetics and Efficiency of Ru-C₉-Ad and 2-Adamantylacetamide Hydroxylation

By supplying electrons to P450 via the natural route (NADH-->PdR-->Rd-->P450), the rate of Ru-C₉-Ad hydroxylation was found to be 10 ± 2 $\mu\text{mol NADH/min}/\mu\text{mol P450}$ (Figure 19). The error in the rate measurement stemmed mostly from the choice of data points--the rate appeared fastest at the earliest time points, as is generally observed for enzyme-mediated catalysis. This rate is remarkably slower than the consumption of NADH in the absence of any substrate (30 $\mu\text{mol NADH/min}/\mu\text{mol P450}$, Figure 19). These data suggest that P450 oxidase chemistry (conversion of O₂ into water) outcompetes hydroxylation of Ru-C₉-Ad threefold. In a control experiment with NADH/PdR/Pd and no P450, the rate was found to be roughly 1 $\mu\text{mol NADH/min}$, thus most of the observed NADH decay may be attributed to P450-mediated catalysis. Product assays indeed show that the yield of hydroxylated product, Ru-C₉-Ad-OH, is only 10% based on the starting NADH concentration. Based on the competing oxidase activity, the maximum predicted yield of hydroxylated product would be ~ 25%. Thus, a 10% product yield is effectively a 40% yield based on the background oxidase activity.

The rate of 2-adamantylacetamide (2) hydroxylation was found to be 90 ± 20 $\mu\text{mol NADH/min}/\mu\text{mol P450}$, in good agreement with the literature value for 1-adamantylacetamide (~ 110 $\mu\text{mol NADH/min}/\mu\text{mol P450}$) (J. J. D. Voss, and P. R. Ortiz de Montellano, *J. Am. Chem. Soc.* **117**, 4185-4186 (1995)). This hydroxylation rate is roughly ten times faster than that of Ru-C₉-Ad, which is not surprising in light of the fact (2) produces a 95% spin conversion of the heme, compared to only 25% for Ru-C₉-Ad. The hydroxylation of Ru-C₉-Ad may also be slowed by the conformational changes observed in Asp 251 and Thr 252, which could affect the delivery of protons leading to dioxygen scission.

The ratio of product formation/NADH consumption with 2-adamantylacetamide is only $47 \pm 3\%$, however, which is half that reported for 1-adamantylacetamide (J. J. D. Voss, and P. R. Ortiz de Montellano, *J. Am. Chem. Soc.* **117**, 4185-4186 (1995)). Thus, these results show that hydroxylation of the model compound, 2-adamantylacetamide, while much faster than Ru-C₉-Ad, occurs with roughly the same efficiency when the background oxidase activity has been subtracted.

Light-activated Ru-C₉-Ad Hydroxylation

Having established that Ru-C₉-Ad turnover is possible via the biological electron transfer route, numerous attempts were made to effect enzyme turnover through steady-state visible irradiation of P450:Ru-C₉-Ad in the presence of *para*-methoxydimethylaniline and dioxygen. Precautions were taken to minimize photodegradation: a 450 nm cutoff filter was used to shield the sample from UV light, and most experiments were conducted at 4 °C to minimize sample heating and to decelerate P420 formation. In addition, experiments were conducted in the presence of catalase to eliminate H₂O₂ which would both damage the enzyme and hydroxylate adamantane, giving a false positive result. Preliminary results suggested that oxidized products are formed in this reaction (Figure 20).

Energy-Transfer Measurements Identifying a P450:Camphor:Ru-C₁₀ Ternary Complex

Titration of camphor into a stoichiometric (5 µM) solution of P450 and Ru-C₁₀ was monitored by UV-vis and energy-transfer measurements, as described in Examples I and II. The binding of camphor to this P450:Ru complex ($K_D = 3.6 \pm 0.2$ µM) is virtually the same as that observed for P450 alone ($K_D = 3.1 \pm 0.2$ µM, Chapter 3) (E. J. Mueller, et al., in *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd edn P. R. Ortiz de Montellano, Ed. (Plenum Press, New York, 1995) pp. 83-124). Additionally, a full low-to-high spin conversion of the heme group is observed upon camphor titration. These results indicate that Ru-C₁₀ does not perturb camphor binding at the active site.

Confirmation of ternary complex formation came from Ru^{2+} decay profiles at varying camphor concentrations which showed only modest changes in the fraction of quenched Ru^{2+} emission. The "two-substrate ternary binding model" is shown in Figure 21.

- 5 The persistence of roughly 25% bound (quenched) $[\text{Ru-C}_{10}]^{2+}$ at high camphor concentrations represents half of the originally bound Ru ($\sim 50\%$, $K_D \sim 2 \mu\text{M}$), and indicates that the affinity of Ru- C_{10} for the P450:camphor complex ($K_D \sim 10 \mu\text{M}$) is not greatly different than for P450 alone. The presence of three distinct quenching processes became evident upon fitting the emission profile to a tri-exponential decay; constraining
10 the fit to three distinct rate constants improved the fitting considerably and gave the consistently best χ^2 values. Weighting the relative coefficients gave the percentage of Ru- C_{10} in each phase (Figure 22).

Kinetics and Efficiency of Camphor Hydroxylation in the Ternary Complex

15

- The rate of NADH consumption was found to be $\sim 600 \mu\text{mol NADH/min}/\mu\text{mol P450}$, in good agreement with literature values (W. A. Atkins, and S. G. Sligar, *J. Am. Chem. Soc.* **109**, 3754-3760 (1987); P. J. Loida, and S. G. Sligar, *Biochemistry* **32**, 11530-11538 (1993)). The addition of Ru- C_{10} (10 equivalents relative to enzyme) was found to
20 decrease the rate of hydroxylation to approximately $300 \mu\text{mol NADH/min}/\mu\text{mol P450}$. Further experiments with varying concentrations of Ru- C_{10} will determine the extent to which the Ru-substrate inhibits camphor turnover.

Discussion

25

- The result that the yield of hydroxylated 2-adamantylacetamide is only half that observed with 1-adamantylacetamide may shed insight on the positioning of critical residues at the active site. While only a small perturbation of the substrate, moving the amide may greatly affect its ability to hydrogen bond with Asp 251 and Thr 252. The movement of
30 these amino acids has been shown to stabilize water molecules at the active site (Dmochowski, I. J. et al., *Proc. Natl. Acad. Sci. USA* (1999) **96**, 12987-12990; I.

Schlichting, et al., *Science* **287**, 1615-1622 (2000)) and may affect the delivery of protons necessary for efficient coupling. Repositioning the amide of Ru-C₉-Ad would be predicted to double the yield of hydroxylation. The design of bulkier Ru-substrates which displace water from the pocket in the open conformation should also promote hydroxylation chemistry. For example, methylation of the sensitizer (i.e., employing 4,4',5,5'-tetramethyl-2,2'-bipyridyl ligands), methylation of the linker (i.e., *para*-xylyl spacers), or increasing the volume of the substrate (i.e., by alkylation) should serve to fill the open hydrophobic pocket and exclude water.

P450 hydroxylates most substrates, including adamantane, much more slowly than camphor, most likely because solvation of the heme lowers the reduction potential for the first electron transfer. In most cases, ET is rate limiting in the substrate hydroxylation reaction. The fact that camphor displaces water and converts the heme to high spin in the presence of Ru-C₁₀ indicates that the reduction potential should be unchanged in the ternary complex. The factors controlling the overall efficiency of the hydroxylation reaction are not completely understood. Almost 100% coupling and high stereoselectivity in the reaction with camphor has been attributed to the substrate's tight binding ($K_D \sim 1 \mu\text{M}$), a hydrogen bond with Tyr 96 and hydrophobic contacts with the protein that orient the molecule correctly.

While not wishing to be bound by any theory, the finding that the rate of NADH consumption during camphor hydroxylation was roughly halved in the ternary complex may be due to 1) changes in the reduction potential of the heme; 2) greater solvent access leading to uncoupling; or 3) inactivation of the enzyme in the ternary complex. Monitoring NADH consumption as a function of the concentration of ternary complex (which is a function of Ru-C₁₀ concentration), as well as quantification of the uncoupling yields will elucidate this rate discrepancy.

It is interesting to note that the conditions for achieving crystallization of the open enzyme - high ionic strength (100 mM NaOAc, 200 mM NH₄OAc), and low solvent dielectric (~ 10% PEG 8000) - have been shown in other work to destabilize the salt

bridges holding the F-G loop (Deprez, E. et al., *Biochemistry* (1994) 33, 14464-14468; V. Lounnas, and R. C. Wade, *Biochemistry* 36, 5402-5417 (1997)). “Loosening” the channel in this way has been shown to be similar to changing Asp 251 to asparagine (C. DiPrimo, et al.. Hoa, *Biochemistry* 36, 112-118 (1997)). Normally, P450 is crystallized in the presence of 0.25 M KCl, with potassium stabilizing the substrate bound in the closed conformation (C. DiPrimo, et al.. Hoa, *Biochemistry* 36, 112-118 (1997)). Therefore, replacement of K^+ with Na^+ , should disfavor the closed conformation. Furthermore, the finding that the crystals nucleated best at 4 °C mirrors the thermodynamics of camphor binding to the Asp251Asn; the association constant for camphor ($\ln K_{eq} \sim 14.2$) decreases above 15 °C for this mutant. This behavior is in marked contrast to WT P450, in which the association constant for camphor ($\ln K_{eq} \sim 14.2$) decreases *below* 15 °C (B. W. Griffin, and J. A. Peterson, *Biochemistry* 11, 4740-4746 (1972)). The perturbation of the bifurcated salt bridge upon Ru-C₉-Ad binding changes the thermodynamics of substrate binding and appears to dictate the conditions under which the complex crystallizes.

The results show that open P450 structures hydroxylate both natural and unnatural substrates with appreciable rates and yields. This example represents the first study of P450 in this solvent-exposed conformation, and may shed light on important mechanistic questions regarding amino acid conformational changes and the delivery of protons associated with dioxygen activation. Although the enzyme is optimized for the substrate camphor, P450 exhibits considerable flexibility in the range of molecules it binds and oxidizes.

25 In addition, these energy-transfer studies have identified one of the first examples of a
ternary complex in cytochrome P450. Energy transfer provides a sensitive tool for
studying complex P450:substrate interactions in solution. This technique can yield
significant structural information about P450 active sites in the absence of a crystal
structure. The large size and hydrophobic nature of P450 substrate channels makes
30 multiple substrate binding a likely event during catalysis.

EXAMPLE IV

This example describes the submillisecond Photooxidation and Reduction of Cytochrome P450 via Sensitizer-Linked Substrates.

5

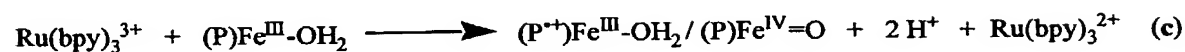
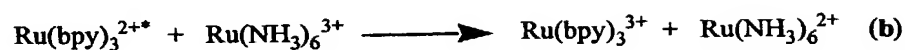
Presented in this example is a new photochemical method for the delivery of both electrons and holes to buried redox sites. By tethering a Ru-photosensitizer to a protein substrate, reducing the P450 heme has occurred much more rapidly than has been possible previously, and a hitherto unobserved oxidized state of the enzyme has also been
10 generated. The strategy of linking sensitizers to substrates opens the door to exploration of reactive redox states in enzyme interiors.

Rigorous characterization of metalloenzyme oxidation states is essential to understanding metabolic processes at a molecular level. Reactive intermediates in enzymatic catalysis
15 are of special interest, but they are frequently too short-lived to be examined directly. A case in point is the high-valent heme that is believed to be a catalytic intermediate in the oxygenation reactions of cytochrome P450_{cam} (P450) (M. Sono, et al., *Chem. Rev.* **96**, 2841-2887 (1996); E. J. Mueller, et al., in *Cytochrome P450. Structure, Mechanism, and Biochemistry* P. R. Ortiz de Montellano, Ed. (Plenum Press, New York, 1995), pp. 83-
20 124). This oxidant has eluded detection thus far, raising questions concerning its role in the catalytic cycle (M. Sono, et al., *Chem. Rev.* **96**, 2841-2887 (1996); L.-L. Wong, *Curr. Op. Chem. Biol.* **2**, 263-268 (1998)).

It has previously been shown that reactive high-valent hemes of peroxidases can be
25 prepared in solutions containing photogenerated ruthenium-diimine oxidizing agents (J. Berglund, et al., *J. Am. Chem. Soc.* **119**, 2464-2469 (1997); D. W. Low, et al., *J. Am. Chem. Soc.* **118**, 117-120 (1996)). Formation of [Ru(bpy)₃]³⁺ via the flash-quench approach oxidizes the heme of both horseradish peroxidase (HRP) and microperoxidase-8

30

Equation 1:



(MP-8) in less than a microsecond (Eq. 1abc). In both heme systems, electron transfer is observed to occur from the iron to the oxidized porphyrin (Eq. 1c). HRP, however, achieves a twice oxidized state after reaction with a second equivalent of $\text{Ru}(\text{bpy})_3^{3+}$. This compound I, $(\text{P}^+)\text{Fe}^{\text{IV}}\text{=O}$, species can be observed either under steady-state conditions, or be generated in a time resolved fashion by starting with the ferryl, compound II species (Figure 23).

It was described previously (Example I-III) that the photosensitizer $[\text{Ru}(\text{bpy})_3]^{2+}$ is linked by a hydrocarbon chain to a species with high affinity for the P450 heme pocket: imidazole (Im), adamantane (Ad) or ethyl benzene (EB) (Figure 24). The synthesis of Ru-EB is typical: reaction of thionyl chloride with 10-bromodecanoic acid, followed by addition of 4-ethylaniline, gives the corresponding amide. Addition of the amide to a solution of 4,4'-dimethyl-2,2'-bipyridine and lithium diisopropylamide yielded the derivatized bpy, which was reacted with $[\text{Ru}(\text{bpy})_2\text{Cl}_2]$ to give Ru-EB. Imidazole may ligate the heme iron directly (J. H. Dawson, et al., *J. Biol. Chem.* **257**, 3606-3617 (1982)), whereas the substrates adamantane (R. E. White, et al., *Arch. Bioch. Biophys.* **228**, 493-502 (1984)) and ethyl benzene (D. Filipovic, et al., *Biochem. Biophys. Res. Comm.* **189**, 488-495 (1992)) bound strongly to the hydrophobic active site cavity. Adamantanone displaced ligated water from the enzyme active site, $[\text{P}_{\text{cys}}\text{Fe}^{3+}\text{-OH}_2]$ (P_{cys} is the cysteine thiolate-ligated protoporphyrin IX of P450), to yield five-coordinate $[\text{P}_{\text{cys}}\text{Fe}^{3+}]$ (R. Raag, T. L. Poulos, *Biochemistry* **30**, 2674-2684 (1991)). Ethyl benzene binding, in contrast, leaves the six-coordinate resting state relatively unperturbed.

Addition of stoichiometric Ru-Ad to ferric P450 shifted the Soret absorption maximum from 417 to 415 nm and created a shoulder at 391 nm, indicating binding of the adamantyl moiety in the heme region (all studies were performed under an argon atmosphere with 10 μ M ruthenium complex, 10 μ M enzyme in 100 mM potassium chloride, 20 mM potassium phosphate buffer at pH 7.4 and room temperature.). This peak shift is attributed to lengthening of the Fe^{III}-OH₂ bond or partial water displacement from the [P_{cys}Fe³⁺-OH₂] resting state, both of which accompany the binding of adamantyl compounds in the heme cavity (T. L. Poulos, et al., in *Cytochrome P450. Structure, Mechanism, and Biochemistry* P. R. Ortiz de Montellano, Ed. (Plenum Press, New York, 1995), pp. 125-150). In addition to this absorbance change, there was a decrease in the excited state (Ru²⁺-Ad) lifetime (Excitation at 480 nm (20-ns pulse width); the experimental setup has been described, D. W. Low, et al., *J. Am. Chem. Soc.* **118**, 117-120 (1996)) that has been attributed to quenching by a Förster energy-transfer process. The normally monophasic luminescence decay profile of Ru²⁺-Ad ($k_{1\text{decay}} = 2.2 \times 10^6 \text{ s}^{-1}$) becomes biphasic ($k_{1\text{decay}} = 2.2 \times 10^6 \text{ s}^{-1}$, $k_{2\text{decay}} = 7.7 \times 10^6 \text{ s}^{-1}$) in the presence of P450, with the faster phase accounting for 77% of Ru²⁺ quenching. The rapid luminescence decay ($k_{2\text{decay}}$) has been attributed to Ru-Ad-P450 interaction; the dissociation constant (K_D) is 0.69 μ M for the complex between Ru-Ad and P450. Soret shifts from addition of the ruthenium complexes to P450 were small. Competitive binding assays of camphor and Ru-Ad with P450 by Soret absorption shifts yielded a K_D value of 0.68 μ M.

The Soret absorption maximum of ferric P450 in the presence of equimolar Ru-Im shifted from 417 to 420 nm, indicating ligation of the imidazole by the heme iron (J. H. Dawson, et al., *J. Biol. Chem.* **257**, 3606-3617 (1982)). Luminescence decay of the Ru²⁺-Im:P450 complex was also biphasic ($k_{1\text{decay}} = 2.2 \times 10^6 \text{ s}^{-1}$, $k_{2\text{decay}} = 7.0 \times 10^6 \text{ s}^{-1}$). Approximately 68% of Ru²⁺ quenching occurred via the faster, energy-transfer, phase ($k_{2\text{decay}}$); $K_D = 1.5 \mu$ M for Ru-Im:P450.

A 1:1 mixture of Ru-EB and P450 did not display an altered Soret absorption maximum; however, Ru²⁺-EB was quenched in the presence of P450. The faster of two decay processes ($k_{1\text{decay}} = 2.2 \times 10^6 \text{ s}^{-1}$, $k_{2\text{decay}} = 1.2 \times 10^7 \text{ s}^{-1}$) accounted for 70% of Ru²⁺-EB decay, indicating $K_D = 1.0 \text{ } \mu\text{M}$ for the Ru-EB:P450 complex. Addition of excess camphor ($K_D \sim 1 \text{ } \mu\text{M}$) (E. J. Mueller, et al., in *Cytochrome P450. Structure, Mechanism, and Biochemistry* P. R. Ortiz de Montellano, Ed. (Plenum Press, New York, 1995), pp. 83-124) to Ru-EB:P450 displaces the Ru-linked substrate completely, as judged by an increased contribution of the slower luminescence decay process ($k_{1\text{decay}}$). It is concluded that all three sensitizer-linked substrates bound tightly to the active site of P450.

Methods And Materials

General

Cytochrome P450_{cam} and the Ru-substrates and reductive quenchers were prepared as described, in Example I *supra*. Highly purified ($R_z > 1.4$), decamphored P450 was stored at $-70 \text{ }^\circ\text{C}$ and thawed just before use. Distilled water was further purified by a Barnstead Nano-Pure system. Tris(2,2'-bipyridine)ruthenium(II) chloride and cobalt (III) pentammine chloride (Strem) were used as received. Hexaammineruthenium(III) chloride (Strem) was recrystallized from a minimum of warm hydrochloric acid. The reductive quencher, *para*-methoxy-*N,N*-dimethylaniline (*p*-MDMA), was sublimed (at $30 \text{ }^\circ\text{C}$) and stored sealed under argon in a refrigerator. Precautions were taken to avoid exposing the quencher to light, oxygen, and heat. Periodically, either sublimation or recrystallization of *p*-MDMA from warm water was performed to restore the purity of the white solid. Static absorption spectra were recorded on an HP-8452A spectrophotometer. Steady-state photolysis experiments were conducted with an Oriel 75 watt halogen lamp.

Transient Absorbance Spectroscopy

Solution experiments were performed in sealed cuvettes with P450 and Ru-substrate in 100 mM KCl and 20 mM KPhos buffer, pH 7.4. Samples were fitted with a magnetic stir

bar and deoxygenated by repeated evacuations on a vacuum line followed by backfilling with purified argon (3 x 10 cycles). The quenchers, cobalt (III) pentammine chloride and *p*-MDMA are poorly soluble in aqueous buffer at 5 mM and 10 mM, respectively, and required considerable stirring to dissolve.

5

All samples were excited with either a XeCl excimer (Lambda-Physik LPX 210i, 308 nm)-pumped dye laser (Lambda-Physik FL 3002, 25-ns FWHM) with coumarin 480 dye (Exciton, 480 nm) or a tunable (220-2000 nm, excitation at 480 nm) optical parametric oscillator (Spectra Physics, MOPO) pumped by a frequency-tripled Q-switched Nd:YAG laser (Spectra Physics, 355 nm, 350 mJ/pulse, 8-ns FWHM). The YAG fired continuously at 10 Hz; thus, for longer time base experiments (> 50 ms) software was written to control the opening and closing of a shutter to select desired pulses.

The laser output was attenuated with a polarizer as needed to give 1-2 mJ/pulse at the sample. Laser shots with energies differing by more than 10% from the mean value (laser pulses detected by a photodiode and selected by a discriminator, Phillips Scientific Model 6930) were rejected. The probe light for single-wavelength transient absorption measurements was provided by a 75 watt continuous-wave arc lamp (PTI model A 1010) and focused on the entrance slit of an ISA double 0.1 meter monochromator. For time bases $\leq 100 \mu\text{s}$, increased light intensity and, correspondingly, higher signal to noise was achieved by pulsing the lamp synchronously with the laser excitation (generally 10 Hz). Counter-propagating pump and probe beams were aligned on the sample cuvette. Signal was detected by a Hamamatsu photomultiplier tube (R928); the output signal amplified, digitized (Sony/Tektronix digitizer, Model RTD710A), and recorded on a PC. For time bases 5 μs -1 ms (1024 points/data array), a high-speed (200 MHz) current to voltage amplifier built at Brookhaven National Laboratory was used. For time bases $\geq 1 \text{ ms}$, a slow amplifier (PSD Corp.) was used. Kinetics data are averages of at least 250 laser shots. Transient absorption traces were typically fit to mono- or biexponential functions ($y = c_0 + c_1 e^{-(k_{\text{en}} + k_0)t} + c_2 e^{-k_0 t}$) using the least-squares fitting program *KinfIt*.

30

For multiwavelength transient absorption experiments (Figure 25), probe light was provided by either a microsecond or nanosecond flash lamp powered by the discharge of a variable number of capacitors. The probe beam was delivered by a short fiber optic cable to a beam splitter; roughly 10% of the light was reflected and focused on a fiber optic leading to the reference channel of the diode array detector. The remaining probe light was focused onto a fiber optic directed towards the sample, and made coincident with the pump beam. The probe light was collected by f/2.5 mirrors (bored at the center with small holes for passage of the laser beam) and focused on a fiber optic cable. The fibers containing the reference and probe channels were vertically aligned (3.5 mm apart) and the beams focused onto the entrance slit of the monochromator (SPEX 270M).

Efficient light collection at the sample necessitated minimization of laser scatter by focusing and collimating the pump beam. Particularly in instances where it was important to measure absorbance changes at wavelengths near the laser line, extra probe light was directed at the sample and then filtered (with any scattered laser light) before the monochromator. $[\text{Ru}(\text{bpy})_3]^{2+}$ (MLCT centered at 456 nm) was generally excited between 480 and 490 nm to minimize spectral overlap (and therefore, collected scatter) during observations in the Soret (380-450 nm). Intensities of probe and reference beams were determined by a diode array detector that was controlled by a Princeton Instruments (model ST-116) instrument and interfaced to a PC using commercial data collection software (*Winspec*). The time resolution was set by delaying the probe pulse (after the laser fire) using a signal delay generator (EG&G). Without a programmed delay, the microsecond flash lamp fired $14 \pm 14 \mu\text{s}$ after the laser, as determined by an oscilloscope. For all but the longest time bases ($> 1 \text{ s}$), the 10 Hz laser pulses emitted from the YAG were intercepted from the MOPO at 1 Hz (with the variable-delay shutter described above).

Data were collected and arithmetically manipulated by running a homemade *WinSpec* macro to control the position of a shutter and the timing sequence of blank, excitation, and intermittent stirring cycles. The position of the monochromator and the grating (either 300 or 1200 grooves/mm) was set with a *LabView* routine. Due to the changing

dispersion of light with wavelength, it was necessary to calibrate the x (wavelength) axis by sticking at least three different interference filters in front of the probe light and measuring their peak intensities as recorded by the diode array detector. A calibration function for the diode array was generated by fitting these peaks to the known interference wavelengths. Likewise, offset problems on the y axis (most problematic for data sets with small OD changes) were normalized from the intensities collected during single-wavelength experiments.

Results

Laser excitation of $\text{Ru}^{2+}\text{-Im}$ followed by reductive quenching with *p*-MDMA (G. A. Mines et al., *J. Am. Chem. Soc.* **118**, 1961-1965 (1996)) yields a powerful reductant, $[\text{Ru-Im}]^+$ ($E^0 = -1.24$ V vs. NHE). Reductive quenching of $[\text{Ru}^{\text{II}}(\text{bpy})_3]^{2+}$ yields a ruthenium complex containing a coordinated bpy anion radical, $[\text{Ru}^{\text{II}}(\text{bpy})_2(\text{bpy}^{\cdot-})]^+$ (K. Kalyanasundaram, *Photochemistry of Polypyridyl and Porphyrin Complexes* (Academic Press Ltd., London, 1992), p. 108). Reduction potentials are for $[\text{Ru}(\text{bpy})_3]^{2+}$ (K. Kalyanasundaram, *Photochemistry of Polypyridyl and Porphyrin Complexes* (Academic Press Ltd., London, 1992), p. 144). The E^0 values of derivatives with substrate-terminated hydrocarbon chains should be similar.

In the presence of P450, $[\text{Ru-Im}]^+$ is converted rapidly to $\text{Ru}^{2+}\text{-Im}$. Concomitant with this $[\text{Ru-Im}]^+$ oxidation is heme reduction, as evidenced by a Soret shift from 420 to 445 nm (J. H. Dawson, et al., *J. Biol. Chem.* **257**, 3606-3617 (1982); J. H. Dawson, et al., *J. Biol. Chem.* **258**, 13637-13645 (1983)). The rate constant (k_{ET}) for $[\text{Ru-Im}]^+ \text{Fe}^{3+}$ electron transfer is $2 \times 10^4 \text{ s}^{-1}$ (Figure 26). Similar kinetics ($k_{\text{ET}} = 2 \times 10^4 \text{ s}^{-1}$) were observed upon reductive quenching of the $\text{Ru}^{2+}\text{-EB:P450}$ complex; a 417 to 390 nm Soret shift results from the $[\text{Ru-EB}]^+ \text{Fe}^{3+}$ reaction (Figure 27). Reductive quenching of the $\text{Ru}^{2+}\text{-Ad:P450}$ complex yielded spectroscopic changes comparable to those seen for $\text{Ru}^{2+}\text{-EB:P450}$. This blue-shifted Soret indicates that the reduction product is the

previously unobserved $[\text{P}_{\text{cys}}\text{Fe}^{2+}\text{-OH}_2]^-$ form of P450 (Figure 27) (the $[\text{P}_{\text{his}}\text{Fe}^{2+}\text{-OH}_2]$ form of myoglobin exhibits a Soret band 15-nm blue-shifted from $[\text{P}_{\text{his}}\text{Fe}^{2+}]$ (D. C. Lamb, V. Prusakov, N. Engler, A. Ostermann, P. Schellenberg, F. G. Parak, G. U. Nienhaus, *J. Am. Chem. Soc.* **120**, 2981-2982 (1998)). Typical bulk reduction of P450

5 results in five-coordinate $[\text{P}_{\text{cys}}\text{Fe}^{2+}]^-$ with a Soret peak at 408 nm. The product of $[\text{P}_{\text{cys}}\text{Fe}^{3+}\text{-OH}_2]$ reduction by $[\text{Ru-EB}]^{1+}$ displayed a Soret peak at 390 nm.). Further confirmation of heme reduction was the production of $[\text{P}_{\text{cys}}\text{Fe}^{2+}\text{-CO}]^-$ ($\lambda_{\text{max}} = 446$ nm) upon steady-state visible irradiation of $\text{Ru}^{2+}\text{-EB:P450}$ in the presence of *p*-MDMA and carbon monoxide. The relatively high rate of heme reduction in the photogenerated $[\text{Ru-EB}]^+:\text{P450}$ complex shows that a direct bond to the iron is not required for efficient Ru-

10 heme electronic coupling. Indeed, electron tunneling to the P450 active site via Ru-linked ethyl benzene is over two orders of magnitude faster than reduction by putidaredoxin ($k \sim 50 \text{ s}^{-1}$), a natural redox partner (M. J. Hintz, et al., *J. Biol. Chem.* **257**, 14324-14332 (1982)). The efficient coupling of the sensitizer-linked substrate to the

15 heme can be exploited to generate a high-valent state of the enzyme.

Oxidative quenching of the photoexcited species $\text{Ru}^{2+*}\text{-EB}$ by $[\text{Co}(\text{NH}_3)_5\text{Cl}]^{2+}$ yielded $\text{Ru}^{3+}\text{-EB}$, a strong oxidant ($E^\circ = 1.26 \text{ V}$ vs. NHE) (J. Berglund, et al., *J. Am. Chem. Soc.* **119**, 2464-2469 (1997); Reduction potentials are for $[\text{Ru}(\text{bpy})_3]^{2+}$ (K. Kalyanasundaram,

20 *Photochemistry of Polypyridyl and Porphyrin Complexes* (Academic Press Ltd., London, 1992), p. 144). The $\text{Ru}^{3+}\text{-EB:P450}$ complex undergoes heme to Ru^{3+} electron transfer ($k_{\text{ET}} = 6 \times 10^3 \text{ s}^{-1}$), yielding an oxidized product with a Soret peak at 390 nm (Figure 28). This absorption change is not observed upon laser photolysis of the $\text{Ru}^{2+}\text{-EB:P450}$ complex in the absence of $[\text{Co}(\text{NH}_3)_5\text{Cl}]^{2+}$. A comparison of diode array spectra (Figure

25 29) showing WT and Y96F photooxidation (1 ms after laser excitation) suggests that Tyr 96 scavenges $[\text{Ru-EB}]^{3+}$, resulting in smaller yields of oxidized WT P450. The oxidized species could be a porphyrin π -cation radical, $[\text{P}^+\text{-cysFe}^{3+}\text{-OH}_2]^+$, or an iron^{IV} species, $[\text{P}_{\text{cys}}\text{Fe}^{4+}\text{-OH}_2]^+$. The blue-shifted Soret band in the spectrum of the oxidized heme

accords with the radical formulation; the cysteine thiolate ligand could stabilize an Fe^{IV} state of P450. Hydrogen bonding to this thiolate (T. L. Poulos, B. C. Finzel, A. J. Howard, *J. Mol. Biol.* **195**, 687-700 (1987)), however, decreases the donor strength. Although the reaction product of iodosobenzene and P450 exhibits a 393 nm Soret band (R. C. Blake II, M. J. Coon *J. Biol. Chem.* **264**, 3694-3701 (1989)), the Soret of chloroperoxidase red-shifts upon ferryl formation (R. Nakajima, I. Yamazaki, B. W. Griffin *Biochem. Biophys. Res. Comm.* **128**, 1-6 (1985)). The blue-shifted Soret band exhibited by the heme in the oxidized Ru-EB:P450 complex is not unlike that of P420, a common P450 decomposition product (S. A. Martinis, S. R. Blank, L. P. Hager, S. G. Sligar, G. H. B. Hoa, J. J. Rux, J. H. Dawson *Biochemistry* **35**, 14530-14536 (1996)). Formation of P420, however, is largely irreversible, whereas oxidized Ru-EB:P450 returns to the resting state without appreciable decomposition; indeed, porphyrin π -cation radicals often display Soret bands that are blue-shifted from those of resting hemes (P. Gans, et al., *J. Am. Chem. Soc.* **108**, 1223-1234 (1986); R. H. Felton, et al., *J. Am. Chem. Soc.* **93**, 6332-6334 (1971); A. Wolberg, and J. Manassen, *J. Am. Chem. Soc.* **92**, 2982-2991 (1970)).

The same rate of P450 reduction was observed with Ru-Im, Ru-Ad, and Ru-EB. The ET rate is relatively insensitive to chain length for the Ru-(CH₂)₉₋₁₃ series. One possibility that would explain both sets of anomalous data is that ET occurs through the protein, rather than through the methylene chain. Computer modeling, however, shows no well-coupled pathway from the top of the channel to the heme. Additionally, it is observed that the yield of electron injection from the shorter [Ru-C₇-EB]⁺ to the heme is markedly smaller (and the rate is an order of magnitude slower), suggesting that electrons, in fact, tunnel through the methylene chain (and through space) to the heme.

While not wishing to be bound by any theory, the observed reduction rate may be dominated by the back electron transfer between [Ru(bpy)₃]⁺ and *p*-MDMA^{•+} ($k_q \sim 4 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$). The observed rate and yield of the forward reaction (Ru⁺-EB Fe³⁺ → Ru²⁺-EB Fe²⁺) correlates with the yield of Ru⁺ generated ($\epsilon = 12,000 \text{ M}^{-1}\text{cm}^{-1}$ for Ru(bpy)⁺ and $\epsilon = 8000 \text{ M}^{-1}\text{cm}^{-1}$ for *p*-MDMA^{•+} at 510 nm). Because the yield of Ru⁺-EB Fe³⁺ varies

with laser power, the quencher concentration, and the accessibility of $\{\text{Ru}(\text{bpy})_3\}^{2+*}$ to the quencher (each Ru-substrate binds slightly differently), it is extremely difficult to draw comparisons between compounds. However, qualitatively, the yields of ET products appear to be highest with Ru-C₉-EB and Ru-C₁₀-EB and decrease by roughly a factor of 10 as the chain length is shortened ($-(\text{CH}_2)_7$) or lengthened ($-(\text{CH}_2)_{13}$).

Low yields, and possibilities for P450 photodegradation by the oxidative flash/quench chemistry make it unlikely that high-valent intermediates will be accessible to study by this route (Figure 30). Additionally, P450 has many tyrosines and tryptophans that compete with the heme for the highly oxidizing Ru^{3+} . Prospects for generating reactive intermediates via the reductive route (Figure 30) appear much more promising. Improvements in the design of Ru-substrates (conjugated linkers, optimized driving forces and chain lengths) will permit electron transfer to P450 in the sub-microsecond regime. Such advances are necessary to observe short-lived intermediates, but also to study P450 in the presence of dioxygen, since O_2 reacts rapidly with photoinduced reducing agents to form superoxide. Rapid electron injection into oxy P450 should allow the observation of reactive intermediates on the catalytic pathway.

By employing sensitizer-linked substrates, new oxidized and reduced states of P450 were been prepared (Figure 31). These flash/quench methods provide a wide time window to study highly reactive forms of the enzyme. Both $[\text{P}_{\text{cys}}\text{Fe}^{\text{II}}\text{-OH}_2]^-$ and $[\text{P}^+\text{-cysFe}^{\text{III}}\text{-OH}_2]^+$ are formed in ~ 0.1 ms and persist for ~ 100 ms. Improved design of sensitizers, quenchers, linkers, and substrates will lead to even faster electron and hole injection into P450 and other redox-active enzymes.

EXAMPLE V

This example describes Sensitizer-Linked Substrates with conjugated linker sections for Submicrosecond Electron Injection into Cytochrome P450

The development of ruthenium photosensitizers with high affinity for the cytochrome P450_{cam} (P450) active site has established a new method for rapidly modulating enzyme oxidation states. Photoexcitation of P450:Ru-(alkyl linker)-substrate conjugates in the presence of an oxidative or reductive quencher has been shown to effect heme redox chemistry on submillisecond time scales (J. J. Wilker, et al., *Angew. Chem. Int. Ed.* **38**, 90-92 (1999)). Recently, efforts have been focused on accelerating the rate-limiting electron-transfer (ET) step in P450 catalysis on route to generating reactive catalytic species (E. J. Mueller, et al., in *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd edn P. R. Ortiz de Montellano, Ed. (Plenum Press, New York, 1995) pp. 83-124). With this goal, three new *conjugated* sensitizer-linked probes were synthesized (Figure 32). Compound (a) cannot form a covalent bond to the heme; the perfluorobiphenyl moiety most likely occupies the substrate pocket. Luminescence quenching of the excited state of (a) by P450 has been assigned to a purely Förster energy-transfer process. Compounds (b) and (c) may bind the heme iron directly, as judged from a blue shift (417 → 420 nm) in the Soret. Luminescence quenching of the excited states of (b) and (c) by P450 has been assigned to both Förster energy-transfer and electron-transfer processes. Laser excitation of (c) allows direct photoreduction of P450 on *submicrosecond* time scales. These rate enhancements, in the absence of a reductive quencher, greatly extend the utility of this approach for biophysical studies of short-lived enzyme intermediates.

In previous work with *saturated*, alkyl-tethered, sensitizer-linked substrates (I. J. Dmochowski, et al., *Proc. Natl. Acad. Sci. USA* **96**, 12987-12990 (1999)), it was shown that P450 complexation significantly quenches {Ru(bpy)₃}²⁺* by Förster energy transfer, not ET. Due to the shortened Ru excited-state lifetime, even an efficient reductive quencher such as *para*-methoxy-*N,N*-dimethylaniline (*p*-MDMA) (S. Gould, et al., *J. Am. Chem. Soc.* **112**, 9490-9496 (1990); K. Miedlar, and P. K. Das, *J. Am. Chem. Soc.* **104**, 7462-7469 (1982)), generates little of the long-lived, highly reducing ($E^0 = -1.24$ V vs. the normal hydrogen electrode, NHE) (K. Kalyanasundaram, *Photochemistry of Polypyridine and Porphyrin Complexes* (Academic Press, Lmted., London, 1992)) [Ru-substrate]⁺ necessary for P450 reduction. Myriad problems with *p*-MDMA include poor

water solubility, dioxygen sensitivity, ability to displace Ru-substrates from the active site, and intense spectral features in the oxidized state, p -MDMA⁺⁺. Ongoing efforts to photogenerate reactive metalloenzyme intermediates in both solution and protein crystals motivated the design of sensitizer-linked probes capable of injecting electrons more rapidly and without ancillary quenchers.

In this example the emission lifetimes and transient absorption of three similar conjugated compounds (a-c) bound to P450 were examined; these data highlight the importance of two parameters – driving force and pathway - in excited-state electron injection. Substantial differences in binding and rates of reduction accompany subtle steric and electronic changes in the Ru-probes (a-c). The optimized compound, tmRu-biphenF₈-im (c), reduces P450_{cam} with a rate constant almost one million times that of the natural Fe₂S₂ redox partner, putidaredoxin ($k = 50 \text{ s}^{-1}$). Importantly, the hydrophobicity and histidine-mimicking imidazole functionality of Ru-probes such as (b) and (c) should permit studies of many other natural and unnatural enzyme active sites.

General Methods

Absorption spectra were recorded on an HP-8452A spectrophotometer. Steady-state emission measurements were made on an ISS K2 fluorometer exciting at 470 nm and scanning from 500-800 nm. All electrochemical measurements were made using a CH Instruments Electrochemical Workstation interfaced to a PC using CH Instruments software. Time-resolved luminescence, steady-state emission, single-wavelength and diode array transient absorption measurements, and all standard procedures involving sample preparation were performed as described in previous chapters. Unless stated otherwise, all experiments were performed in 50 mM KPi, 100 mM KCl, pH 7.4 buffer.

Syntheses

Compounds (a-c) were synthesized by procedures similar to those described for Ru-substrates and Ru-Im probes (Example I). Addition of excess perfluorobiphenyl to a

solution of 4,4'-dimethyl-2,2'-bipyridine (GFS) and lithium diisopropylamide yields the derivatized bpy. Reacting this compound directly with $[\text{Ru}(\text{bpy})_2\text{Cl}_2]$ yields (a). Otherwise, addition of stoichiometric imidazole and K_2CO_3 in freshly distilled DMSO yields bpy-biphenyl(F_8)-im, which was reacted with either $[\text{Ru}(\text{bpy})_2\text{Cl}_2]$ or $[\text{Ru}(\text{tmbpy})_2\text{Cl}_2]$ to yield (b) and (c), respectively. The syntheses of (d) and (e) are described in full detail in Example I, *supra*.

Electrochemistry

Cyclic voltammetry (CV) was performed at ambient temperature with a normal three-electrode configuration consisting of a highly polished glassy carbon working electrode, a platinum auxiliary electrode, and a standard calomel electrode reference. The working electrode was separated from the reference compartment by a fritted disk. Acetonitrile solutions contained 0.1 M tetrabutylammonium hexafluorophosphate (freshly recrystallized) as the supporting electrolyte. Samples were rigorously bubbled with argon for several minutes prior to data collection. All compounds were studied as their PF_6^- salts. All potentials are reported vs. NHE, using the relationship $E^0(\text{NHE}) = E^0(\text{SCE}) + 242 \text{ mV}$.

$\text{Ru}^{3+/2+}$ and $[\text{Ru}(\text{bpy})_2(\text{bpy}')^{0/+}]^{2+}$ couples for compounds (a), (b), (c), and (e) were recorded and corrected for the junction potential using ferrocenium/ferrocene as an internal standard. In the cell, CV of 0.05 mM ferrocene solution in 0.1 M tetrabutylammonium hexafluorophosphate gave $E^0(\text{Fc}^+/\text{Fc}) = 498 \text{ mV}$ vs. SCE. Junction potentials in acetonitrile were found to be 191 mV.

Results

Binding of Ru compounds to P450 was determined by UV-vis and luminescence lifetime measurements, as described previously (Example I and in J. J. Wilker, et al., *Angew. Chem. Int. Ed.* **38**, 90-92 (1999); I. J. Dmochowski, et al., *Proc. Natl. Acad. Sci. USA* **96**, 12987-12990 (1999)). Addition of equimolar tmRu-biphenF_9 (a) to substrate-free P450

perturbed the absorption spectrum very little (λ_{max} remained at 417 nm). Time-resolved emission experiments, however, showed that P450 binds (a) tightly ($K_D = 0.9 \mu\text{M}$). The normally monophasic Ru^{2+} luminescence profile became biphasic in the presence of P450 ([enzyme] = $4.8 \mu\text{M}$, [Ru] = $4.5 \mu\text{M}$, 65% $k_{\text{bound}} = 1.0 \times 10^7 \text{ s}^{-1}$, 35% $k_{\text{free}} = 2.0 \times 10^6 \text{ s}^{-1}$). The observed decay is attributed to quenching of the bound Ru^{2+} by a Förster energy-transfer process (T. Förster, in *Modern Quantum Chemistry* O. Sinanoglu, Ed. (Academic Press, New York, 1965), vol. III, pp. 93-137), similar to that observed with other Ru-probes (Example I and in I. J. Dmochowski, et al., *Proc. Natl. Acad. Sci. USA* 96, 12987-12990 (1999)). No transient absorption signals other than those associated with the decay of Ru^{2+} were observed for this complex, indicating the absence of an ET process. Based on Förster analysis of the overlap between Ru^{2+} emission and P450 absorption spectra (Figure 34), R_0 is calculated to be 18.8 Å (Table 33). The rate of Ru^{2+} energy transfer ($k_{\text{en}} = k_{\text{bound}} - k_{\text{free}} = 8.0 \times 10^6 \text{ s}^{-1}$) predicts a Ru-Fe distance of 19.7 Å, which agrees well with modeling studies of Ru-biphenF₉ bound to the open P450 structure.

Addition of equimolar Ru-biphenF₈-im (b) to substrate-free P450 red shifted the absorption spectrum ($\lambda_{\text{max}} = 417 - 420 \text{ nm}$), similarly to known Fe³⁺-imidazole P450 complexes (J. H. Dawson, et al., *J. Biol. Chem.* **257**, 3606-3617 (1982)). The Q-bands were also red shifted from ferric-aquo P450 (Figure 34), although less than most ferric-imidazole complexes. The observed biphasic Ru²⁺ luminescence profile ([P450] = [Ru] = 10 μM , 55% $k_{\text{bound}} = 1.2 \times 10^7 \text{ s}^{-1}$, 45% $k_{\text{free}} = 2.0 \times 10^6 \text{ s}^{-1}$) confirmed P450 binding ($K_{\text{D}} = 3.7 \pm 0.2 \text{ }\mu\text{M}$). At early times ($\tau \sim 300 \text{ ns}$) transient absorption signals were observed for not only decaying Ru²⁺ but also increasing Ru³⁺-Im-Fe²⁺ (Figure 35), permitting assignment of Ru²⁺ quenching to both Förster energy-transfer and electron-transfer processes. All of the unbound Ru²⁺ returned to the ground state within 5 μs of laser excitation, and the remaining signal (Figure 36, absorption increase centered at 445 nm, bleach centered at 420 nm) agreed with numerous Fe²⁺-imidazole P450 species (J. H. Dawson, et al., *J. Biol. Chem.* **258**, 13637-13645 (1983)).

Stoichiometric addition of tmRu-biphenF₈-im (c) to substrate-free P450 red shifted the absorption spectrum ($\lambda_{\text{max}} = 417 \rightarrow 420$ nm), similarly to (b). Time-resolved emission experiments showed P450 bound this methylated compound ($K_D = 0.5 \pm 0.2$ μM) considerably better than Ru-biphenF₈-im. The observed biphasic Ru²⁺* luminescence profile ([P450] = [Ru] = 10 μM , 80% $k_{\text{bound}} = 4.0 \times 10^7$ s⁻¹, 20% $k_{\text{free}} = 4.5 \times 10^6$ s⁻¹) (Figure 37) was attributed to quenching of the bound Ru²⁺* by *predominantly electron transfer*. Transient absorption signals were observed for both Ru²⁺* and tmRu³⁺-Im-Fe²⁺ at the earliest times ($\tau \sim 10$ ns) accessible to the BILRC nanosecond laser system (Figure 38), and achieved a maximum Δ absorbance 30 ns after the laser pulse (Figure 39). The product of this direct photoinduced ET appears virtually the same as that observed upon reduction of the P450:(c) complex with dithionite and laser photolysis (Figure 40). This Fe²⁺:imidazole-Ru complex persists for several hours at room temperature without degradation.

15 Direct Photoinduced Reduction

Having established that excited-state ET, Ru²⁺* \rightarrow Fe³⁺, occurs with conjugated Ru-Im probes (b) and (c), it remains to determine the rates of both the forward and back, Fe²⁺ \rightarrow Ru³⁺, ET processes. The observed rate (k_{obs}) at which the ruthenium excited state decays is the sum of three competing first-order processes: ET (k_{ET}), energy transfer (k_{en}), and the intrinsic (radiative + nonradiative) decay to ground state (k_d) (Eq. 6.1).

$$k_{\text{obs}} = k_{\text{ET}} + k_{\text{en}} + k_d \quad (6.1)$$

k_d may be determined by time-resolved emission experiments of the Ru-probes themselves, alone in solution. Since all of the Ru²⁺* emission profiles are biphasic exponential decays in the presence of P450, it is best to subtract the unquenched rate (k_d) from the quenched rate (k_{obs}) in order to get $k_{\text{obs}} - k_d = k_{\text{ET}} + k_{\text{en}}$. Solving for k_{ET} requires finding the yield of reduction based on the concentration of *bound* excited state generated each laser pulse. Based on the dissociation constant for calculated for (b) and (c):

$$\%Ru^{2+*}(\text{bound}) = \%Ru(\text{bound}) \times [Ru^{2+*}] \quad (\text{Eq. 6.2})$$

The dependence of the ET rate on excited-state yield makes such calculations extremely sensitive to the laser power. Power dependence studies gave a good indication of the fraction of the ruthenium excited with each laser shot (Figure 41). It was found empirically that exciting the sample with more than ~ 3.3 mJ/pulse (beam diameter ~ 2 mm) was sufficient to achieve nearly quantitative Ru^{2+*} . Additionally, the ΔOD amplitudes (from fits of kinetics traces) make it is possible to calculate the concentration of Ru^{2+*} at 370 nm ($Ru^{2+*} - Ru^{3+}$, $\Delta\epsilon = 8050 \text{ M}^{-1}\text{cm}^{-1}$, work by I.J. Chang). Calculations of the Fe^{2+} yield can be made at 420 nm ($Fe^{2+}-Fe^{3+}$, $\Delta\epsilon = -82,000 \text{ M}^{-1}\text{cm}^{-1}$), and at 445 nm ($Fe^{2+}-Fe^{3+}$, $\Delta\epsilon = 81,000 \text{ M}^{-1}\text{cm}^{-1}$, once the back reaction is taken into account (Eq 6.3):

$$\frac{\partial(Fe^{II})}{\partial t} = k_{ET}[Ru^{2+*}] - k_{back}[Fe^{2+}] \quad (\text{Eq. 6.3})$$

From Eq. 6.1 and the definition of a first-order rate expression, gives:

$$\frac{\partial(Ru^{2+*})}{\partial t} = k_{obs}[Ru^{2+*}] \quad (\text{Eq. 6.4})$$

which integrates to:

$$[Ru^{2+*}] = [Ru^{2+*}]_0 e^{-(k_{obs}t)} \quad (\text{Eq. 6.5})$$

where $[Ru^{2+*}]_0$ is the initial concentration of *bound* excited-state Ru. Plugging this back into Eq. 6.3, gives:

$$\frac{\partial(Fe^{II})}{\partial t} = k_{ET}[Ru^{2+*}]_0 e^{-(k_{obs}t)} - k_{back}[Fe^{2+}] \quad (\text{Eq. 6.6})$$

Solving the differential equation for Fe^{II} as a function of time gives:

Thus, plugging in the concentration of Fe^{II} and the time (t) yields k_{ET} , since all of the other variables are experimentally measurable quantities. For the P450:(c) complex ([P450] = [Ru] = 8.9 μM ; [unbound Ru] = 1.85 μM , $\Delta\epsilon = -2400 \text{ M}^{-1}\text{cm}^{-1}$ at 445 nm), the maximum ΔOD at 445 nm (observed at 30 ns) was $40 \pm 2 \text{ mOD}$ (Figure 40). Adjusting for the bleach from the unbound Ru^{2+*} at 445, the ΔOD at 445 nm increases to 44.5 mOD. Since $[\text{Ru}^{3+}] = [\text{Fe}^{2+}]$, and $\Delta\epsilon(\text{Ru}) / \Delta\epsilon(\text{Fe}) = 1/9$ at 445 nm, the total absorbance change due to the reduction of iron is adjusted upward to 49.5 mOD, which corresponds to 0.61 μM Fe^{2+} -Im (8.7% yield based on bound Ru). Plugging this concentration (at t = 30 ns) into Eq. 6.7 with a measured back electron transfer rate, k_{back} , of $3 \times 10^7 \text{ s}^{-1}$, and a k_{obs} of $3.9 \times 10^7 \text{ s}^{-1}$, yields an ET rate, $k_{\text{ET}} = 8.1 \times 10^6 \text{ s}^{-1}$. Evaluating Eq. 6.1 gives $k_{\text{en}} \sim 2.6 \times 10^7 \text{ s}^{-1}$, which is a factor of 3 faster than predictions for a Förster energy-transfer process. Judging from the energy-transfer rate and distance found in the P450:(a) complex, k_{en} should be roughly $9.5 \times 10^6 \text{ s}^{-1}$. Using this value, Förster analysis of the overlap between tmRu^{2+*} emission and P450 absorption spectra predicts $R_0 = 22.1 \text{ \AA}$ and a Ru-Fe distance of 17.7 \AA , in good agreement with the crystal structure (Figure 33).

The reason for the discrepancy between calculated and predicted rates stems from one of three reasons: 1) the calculated yield of reduced P450 is only 25% of the true value, 2) the back ET rate is considerably faster ($\sim 1 \times 10^8 \text{ s}^{-1}$) than measured and is poorly resolved in the response time of the instrument, or 3) the majority of Ru^{2+*} decays by a different and unassigned pathway (i.e., Dexter energy transfer). Without further experiments, particularly picosecond transient absorption, and better spectral deconvolution, it will be difficult to discriminate between these alternate explanations.

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Yields of reduction in the P450:Ru-biphenF₈-im complex, observed 5 μs after laser excitation, are $1 \pm 0.5\%$ (based on the fraction of bound Ru) using the procedure described for tmRu -biphenF₈-Im. This yield predicts that the rate of electron transfer is approximately $1 \times 10^6 \text{ s}^{-1}$, and by Förster analysis, $R_0 = 22.1 \text{ \AA}$, and Ru-Fe = 17.7 \AA . The transient absorption kinetics (Figure 35) failed to exhibit the submicrosecond reduction phase observed with (c), but otherwise yielded similar spectral features.

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Interestingly, in P450 complexes of both (b) and (c), the ground state back electron transfer ($\text{Ru}^{3+}\text{-Im-Fe}^{2+} \rightarrow \text{Ru}^{2+}\text{-Im-Fe}^{3+}$) does not return all of the enzyme to its resting ferric state. In fact, three observably different back ET processes whose rates span 6-7
5 orders of magnitude were observed. Since the OD changes are much larger with (c), and the back ET rates are similar for both compounds, our analysis shall focus on the latter.

Any explanation for the three observed back ET rates must be consistent with the observation that the initially formed $\text{Fe}^{2+}\text{-Im}$ P450 species bleaches but does not change
10 its spectral profile (i.e., imidazole remains bound) during the entire back reaction (microseconds to seconds). The diode array spectrum of (c) shows a consistently red-shifted Soret on all time scales (Figure 42). Having excluded, therefore, the possibility of ligand exchange, and considering that bimolecular ET reactions should not compete with the rapid intramolecular ($\text{Ru}^{3+}\text{-im-Fe}^{2+} \rightarrow \text{Ru}^{2+}\text{-im-Fe}^{3+}$) ET, it is most likely that the
15 protein scavenges some of the Ru^{3+} . Indeed, Tyr 29 has recently been implicated in the chiral discrimination of Ru-substrates binding to the P450 channel. Based on the yield of reduced P450 at 5 μs ($\sim 2\%$) relative to 30 ns ($\sim 9\%$), and the predicted back ET rate ($k_{\text{back}} = 3 \times 10^7 \text{ s}^{-1}$), we infer that the Tyr $\rightarrow \text{Ru}^{3+}$ ET reaction must be occurring with a rate constant of $k \sim 6.0 \times 10^6 \text{ s}^{-1}$. The low yields of this reaction make observations of a
20 tyrosine radical ($\text{Tyr}^{\cdot+}$) difficult by transient absorption spectroscopy ($\lambda_{\text{max}} = 420 \text{ nm}$) (Y. Chen-Barrett, et al., *Biochemistry* **34**, 7847-7853 (1995)). Consistent with this explanation, however, is the apparent disappearance of most Ru^{3+} absorbance (observed at 320 nm) by 250 ns. The slower back ET rate ($k = 2 \times 10^4 \text{ s}^{-1}$) is proposed to occur from $\text{Fe}^{2+}\text{-Im} \rightarrow \text{Tyr}^{\cdot+}$. The forward and back ET processes are summarized in Figure
25 43.

Most ($\sim 80\%$) of the remaining $\text{Fe}^{2+}\text{-Im}$ appears, in fact, to decay by this second back ET process, but a small fraction of the reduced P450 persists for ~ 1 second after the laser pulse. Presumably, one of several bimolecular processes is at play – bound and free Ru
30 $^{2+/3+}$ exchange reactions, as well as protein-protein ET reactions. In support of this hypothesis is the finding that the addition of micromolar tmRu (e) to a 1:1 P450:(c)

complex changed the back electron transfer step from a primarily first-order process to a second-order process dependent on the concentration of (e).

No changes in the Soret were observed upon laser excitation of P450 complexed with perfluorobiphenyl imidazole (d). Thus, photodissociation appears to be an unlikely process with any of these compounds. Compound (e) and $[\text{Ru}(\text{bpy})_3]^{2+}$ were not quenched upon addition of P450, indicating that a tether is essential for binding as well as energy/electron transfer. In all cases, sample integrity was monitored by UV-vis before and after each experiment, and no photo-mediated degradation was observed. Binding of the Ru-probes was shown to be fully reversible by displacing the compounds with camphor and returning the enzyme to its 5-coordinate ferric resting state.

To determine whether rapid electron injection from the Ru excited state of (c) to the heme operates by hopping (bipyridyl \rightarrow perfluorobiphenyl \rightarrow heme) rather than tunneling, electrochemistry was performed on both the model compound (e) (Figure 44) and on (c) (Figure 45). The bridging model compound (d) exhibited quasi-reversible waves by CV as observed previously for perfluorobiphenyl (B. H. Campbell, *Anal. Chem.* **44**, 1659-1663 (1972)). The reduction potential of the bridge lies several hundred millivolts below that of the excited state of (a-c), and electron hopping may be discounted as a viable mechanism for electron transfer.

The bulkier, conjugated Ru-probes described in this Example bind extremely well; (c) binds, in fact, better than the saturated Ru-Im probes synthesized previously (Example I). It is likely that the enhanced affinity is driven by hydrophobic interactions, particularly involving the perfluorinated bridge and the addition of 8 methyl groups on the terminal Ru moiety. Evidently, the greater steric bulk does not preclude binding, and raises the question of how close to the heme a slightly smaller photosensitizer (i.e., $[\text{Ru}(\text{CN})_4(\text{bpy})]^{2-}$) might bind the active site. Energy-transfer calculations predict that (b) and (c) bind 2-3 Å closer to the heme than the Ru-probes described in Example I. The rigidity of the conjugated bridge, and also, perhaps, the angle at which the bridge is canted from the bipyridyl ligand (Figure 32), appears to promote smaller Ru-Fe distances.

It is possible that the heme iron does not covalently bond (perfluorobiphenyl)imidazole in the ferric state. The Soret and Q-bands are only modestly red-shifted, with the relative intensities of the α and β bands changing slightly relative to the $\text{Fe}^{3+}\text{-OH}_2$ resting state (Figure 33). This behavior contrasts the binding of imidazole and $\text{Ru-C}_{13}\text{-Im}$ which red shift the Q-bands by several nanometers. In principle, the imidazole could act as a Lewis base and deprotonate $\text{Fe}^{3+}\text{-aquo}$; the modestly red-shifted Soret would then correspond to a $\text{Fe}^{3+}\text{-hydroxy}$ species.

Interestingly, the imidazole-perfluorobiphenyl model compound (d) shows a similar absorption spectrum ($\lambda_{\text{max}} = 420 \text{ nm}$) upon binding P450 in the ferric state. This suggests that the lack of significant absorbance changes may be due to the electron-withdrawing nature of the perfluorobiphenyl bridge rather than the size of these conjugated molecules. The predicted π -acidity and weakened σ -donating ability of this interesting new imidazole ligand would greatly stabilize lower oxidation states, and, in fact, the standard $\text{Fe}^{2+}\text{-Im}$ spectrum ($\lambda_{\text{max}} = 446 \text{ nm}$) is observed upon reduction (Figure 40). Further support for this hypothesis comes from emission lifetime binding studies of the 1:1 P450:(b) complex; the Ru^{2+} profile is highly quenched by ET and predominantly monophasic, suggesting that the Ru-Im probe is much more tightly bound to Fe^{2+} P450. A strong preference for imidazole in the ferrous oxidation state is quite unusual for heme enzymes, and points to a complex equilibrium in the ferric state. Such ligand exchange processes should be pH sensitive.

The yield of reduced P450 ($\text{Ru}^{2+}/\text{Fe}^{3+} \rightarrow \text{Ru}^{3+}/\text{Fe}^{2+}$) appears to depend heavily on the excited-state driving force as well as the availability of a through-bond covalent pathway to the heme. The addition of eight methyl groups on the bipyridyl ligands lowers the reduction potential by approximately 160 mV which increases both the rate of reduction and yield of ET products by an order of magnitude. With both Ru-Im compounds, the reduction potential of the heme is roughly -300 mV vs. NHE (~50 mV higher than that expected for an imidazole-ligated P450 heme, due to the electrophilicity of perfluorobiphenyl). The excited-state reduction potential of (c) is approximately -1.0 V,

providing substantial driving force for reduction ($-\Delta G \sim 0.7$ V) in a nearly activationless reaction.

The advantage of a directly covalent pathway for excited-state electron transfer is evident from the lack of ET products with (a). This Ru moiety is calculated to bind an additional 2 Å from the heme (Figure 33), which would require a substantial through-space jump from the biphenyl to the iron. One exciting finding with (a) was that it forms a stable ternary complex with either imidazole or carbon monoxide, as shown by energy-transfer measurements. Photoexcitation of the P450:Ru-biphen:imidazole complex yielded reduced (Fe^{2+} -Im) protein, due presumably to either a shorter through-space jump or a lower reorganization energy for $\text{Fe}^{3+/2+}$ -Im than for reduction of the ferric aquo heme. Successful photoinduced ET in this ternary complex bodes well for future studies of dioxygen activation and light-activated substrate turnover.

Accordingly, conjugated sensitizer-linked probes of the invention bound with high affinity and promote rapid electron transfer to the buried P450 heme. Submicrosecond rates of electron injection from Ru^{2+} to the iron agreed with experiment and theoretical predictions for well-coupled ET reactions that are nearly driving force optimized (R. A. Marcus, and N. Sutin, *Biochim. Biophys. Acta* **811**, 265-322 (1985); H. B. Gray, and J. R. Winkler, *Annu. Rev. Biochem.* **65**, 537-561 (1996); A. Helms, et al., *J. Am. Chem. Soc.* **114**, 6227-6238 (1992); W. B. Davis, et al., *Nature* **396**, 60-63 (1998)). Large enhancements of the rate and yield of ET were achieved with the use of tetramethylated bipyridyl ligands attached to the Ru photosensitizer. Quantification of the rate of electron injection was complicated by the difficulty of determining the yield of Fe^{2+} -Im formed in this reaction.

EXAMPLE VI

This example describes $[Ru(phen)_2dppz]^{2+}$ based luminescent probes for Cytochrome P450_{cam}.

The luminescence of $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ complexes when bound to DNA is the result of the protection of the dppz ligand from water by intercalation between the DNA base pairs. (Erkkila, K. E. et al., *Chem. Rev.* (1999) **99**, 2777-2795; Kielkopf, C. L. et al., *Nature Str. Biol.* (2000) **7**, 117-121) In principle, any similar shielding from aqueous environment should lead to luminescence. Accordingly, a series of compounds in analogy to $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ that would luminesce upon binding to cytochrome P450 were synthesized.

Materials and Methods

General: P450cam was overexpressed in *E. coli* and purified as previously described (B. H. Campbell, *Anal. Chem.* **44**, 1659-1663 (1972). Both transient absorption and emission data were collected on instruments already described in the literature. (Low, D. W. et al., *J. Am. Chem.* (1996) **118**, 117-120; **14**) NMR spectra were taken on a General Electron QE300 or Varian Mercury 300. Static absorption spectra were taken on a HP-8452A spectrophotometer. Steady state luminescence spectra were taken on an ISS K2 Fluorometer. Quantum yields were calculated relative to a $[\text{Ru}(\text{bpy})_3]^{2+}$ standard, whose quantum yield was taken to be 0.42. Electrospray mass spec. data was collected on a Finnigan LCQ quadrupole ion trap mass spectrometer.

P450 was stored in small aliquots and thawed immediately before use. Samples were prepared in 50 mM Kphos buffer containing 100 mM KCl. P450 concentration was quantified using the heme soret absorption at 416.5 nm ($115,000 \text{ M}^{-1}\text{cm}^{-1}$). Samples were prepared in a custom quartz cuvette fitted with a Kontes Teflon stopcock. Oxygen was removed from the sample by completing at least 30 cycles of partial vacuum followed by an influx of argon.

Syntheses:

$\text{Ru}(\text{phen})_2\text{Cl}_2$ and $\text{Ru}(\text{tmbpy})_2\text{Cl}_2$ were synthesized as reported. (G. A. Mines, et al., *J. Am. Chem. Soc.* (1996) **118**, 1961-1965). All other reagents were purchased from the

Aldrich Chemical Co. and used as received. THF was dried by refluxing over calcium hydride for at least 3 days, and was then distilled onto activated 3 Å molecular sieves.

Synthesis of Ru(phen)₂dppa-C6-Ad 1. 0.30 g. Ru(phen)₂dppa (0.278 mmol) and
5 0.0972g 6-amino-hexanoic-adamanyl amide (deprotected 5) (0.417 mmol) were mixed in
5 mL dry DMF with 217 mg BOP (0.417 mmol) and 144 mg DIPEA (1.11 mmol). The
reaction was sealed and left to stir for 14 hrs. The crude reaction product was
concentrated under vacuum, and the residue was purified by flash chromatography using
80/10/10 acetonitrile/butanol/water mixture saturated with KNO₃. The fractions which
10 contained product were pooled and concentrated until only butanol remained. The
butanol solution was then diluted 5x with CHCl₃ and filtered to removed KNO₃ and
silica. The filtered solution was then concentrated to yield 142 mg 5 (54.4 %).

Synthesis of Ru(phen)₂dppa-gly-adm: 2 0.190 g Ru(phen)₂dppa (0.176 mmol), 0.107 g
15 6 (0.353 mmol) 183 mg BOP (0.353 mmol) and 0.123 mL DIPEA were dissolved in 3.4
mL anhydrous DMF. The reaction was left to stir overnight. DMF was removed under
vacuum, and the residue purified via flash chromatography using a 70/15/15
acetonitrile/water/95% ethanol mixture saturated with KNO₃ eluent. The fractions
containing product were pooled and concentrated under reduced pressure. The mixture
20 of salts and product was washed with 5x100mL CHCl₃ and the combined organic layers
were concentrated under vacuum. The crude product was dissolved in water and
precipitated with an excess of NH₄⁺PF₆⁻. The precipitate was washed with water,
dissolved in acetonitrile and re-concentrated under vacuum. Yield was 156.5 mg (70 %).

Synthesis of Ru(phen)₂dppa-adm. 3 132.5 g Ru(phen)₂dppa (0.123 mmol) 37.2 mg 2-
25 adamanylamine (0.246 mmol) 128 mg BOP (0.246 mmol) and 0.1 mL DIPEA (0.615
mmol) were dissolved in 2.4 mL anhydrous DMF and sealed in a 10 mL round bottom
flask. The reaction was left to stir overnight. The reaction mixture was concentrated in
vacuo and purified using flash chromatography with a mixture of 80/10/10
30 acetonitrile/water/95 % ethanol saturated with KNO₃ as eluent. The product containing
fractions were concentrated under reduced pressure until only water remained. The

product was then precipitated with excess $\text{NH}_4^+\text{PF}_6^-$, filtered over a fine frit, and washed with deionized water. The product was then dissolved in acetone and re-precipitated with $(\text{nBu})_4\text{N}^+\text{Cl}^-$ collected on a frit, and washed with acetone. Total yield was 65.3 mg (53.6 %).

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Synthesis of Fmoc-protected 6-amino-hexanoic acid 4. 2.572 g Fmoc-succinimdy ester (7.623 mmol) was dissolved in 50 mL dioxane. 1.00 g 6-amino-hexanoic acid (7.623 mmol) was dissolved in 100 mL pH 9 water, and cooled to 0 °C. The Fmoc solution was added dropwise with constant stirring. A white precipitant formed immediately. The reaction vessel was sealed, and left to stir overnight. The reaction was then brought to neutral pH and extracted with 3x50 mL CH_2Cl_2 . Product was isolated in > 95 % yield.

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Synthesis of Fmoc-6-amino-hexanoic-adamanyl amide. 5 1.068 g 4 (3.025 mmol) 0.416 g 2-adamantyl amine (3.33 mmol) 0.460 g HOBT (3.025 mmol) and 0.71 mL DIPCI (4.54 mmol) were dissolved in CH_2Cl_2 and left to stir overnight in a sealed 50 mL round bottom flask. The contents of the flask were concentrated under reduced pressure and purified using flash chromatography with 50/50 ethyl acetate/hexanes increasing to 70/30 ethyl acetate/hexanes. Product eluted slightly after the residual starting material. The fractions containing product were pooled and concentrated under reduced pressure, yielding a white solid. 1.082 g (78.1 %).

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Synthesis of 1,10-phenanthroline-5,6-dione 6. The published synthesis gave inconsistent results.(Yamada, M. et al., *Bull. Chem. Soc. Jpn.* (1992) **65**, 1006) A modified procedure described herein gives more reliable results. Conc. sulfuric acid (40 mL) and conc. (70 %) nitric acid (20 mL) were cautiously, and thoroughly mixed. The mixture was cooled to -20 °C using a dry ice/acetone bath. Phenanthroline (4.00 g) and KBr (4.00 g) were placed in a 250 mL round bottom flask, and cooled in a dry ice/acetone bath. The acid solution was added all at once to the chilled flask, and the reaction vessel placed in an ice/salt bath held at about 0 °C. A reflex condenser was attached, and the reaction was stirred as vigorously as possible. The bath was then heated

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the acetonitrile was removed under reduced pressure. The pH of the resulting aqueous solution was adjusted to 1 with dilute HCl, and the product precipitated with a large excess of $\text{H}_4\text{N}^+\text{PF}_6^-$. The product was isolated by filtration and dried under vacuum. The isolated yield was 0.385g dark red powder (58.3 %). Characterization matched previously reported values.(Hartshorn, R. M. and Barton, J. K. *J. Am. Chem. Soc.* (1992) 114, 5919-5925)

Results and Discussion

The molecules shown in Figure 47 (as depicted, compound 1 is Ru-dppa-C₆-Ad, compound 2 is Ru-dppa-gly-Ad, and compound 3 is Ru-dppa-Ad) were synthesized. In this study, the parent ligand, dipyrido([3,2-a:2',3'c]phenazine (dppz) was modified to include a carboxylic acid moiety. The resulting ligand, 4,5,9,14-tetraazo-benzo[b]triphenylene-11-carboxylic acid, is referred to as dppa in keeping with previous nomenclature.(Hartshorn, R. M. and Barton, J. K. *J. Am. Chem. Soc.* (1992) 114, 5919-5925) The synthesis was performed using standard peptide coupling techniques, as described above (Figure 48). Previous studies had shown that $[\text{Ru}(\text{bpy})_3]^{2+}$ connected to an adamantyl unit by a long alkyl chain bound with sub-micromolar affinity to P450 (Wilker, J. J. et al., *Angew. Chem. Int. Ed.* (1999) 38, 90-92). Thus, it was decided to test the binding of a $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ moiety attached to an adamantyl group by an alkyl chain of varying lengths. All of the compounds synthesized bound with micromolar affinity. Interestingly, 1 ($K_d = 0.25 \mu\text{M}$) and 3 ($K_d = 0.18 \mu\text{M}$) both bound more tightly than 2 ($K_d = 2.4 \mu\text{M}$). All of the compounds synthesized have binding constants that compare favorably to camphor ($K_d = 1.6 \mu\text{M}$).

The dissociation constants of the analogous $\text{Ru}(\text{bpy})_3$ -alkyl-adamantyl compounds, for example compound Ru-C₉-Ad, fall around $0.2 \mu\text{M}$. The observed binding constant of 1 is reasonable in light of these previous results. The reduced binding constant of 2 cannot be explained by steric clashes between the ruthenium complex and the protein, because 3 binds even more tightly than 1. Compound 1 was manually docked into P450 (global change, capitalize P450) frozen in the conformation observed in the crystal structure of

P450 bound to a similar tris-bipyridyl ruthenium complex (Figure 48). (Dmochowski, I. J. et al., *Proc. Natl. Acad. Sci. USA* (1999) 96, 12987-12990) As modeled, there were no significant steric clashes. Further, a large portion of the hydrophobic complex was desolvated, which should lead to a favorable free energy of binding. While not wishing to be bound by any theory, possible explanations for the relatively low binding affinity of compound 2 include: (1) the additional peptide bond may produce an undesirable steric clash; (2) glycine is relatively hydrophilic, and the increased hydrophilicity of 2 in comparison to 1 and 3 should result in a higher K_d .

10 These results indicate that P450_{cam} tolerates large, hydrophobic molecules in the channel leading to the active site.

The luminescence of compounds 1-3 was examined in acetonitrile, potassium phosphate buffer, and buffer in the presence of P450 (as described above). Compound 1 did not show a significant increase in luminescence over background levels. The complexes 2 and 3 show modest recoveries of luminescence, with integrated intensities that are 2.6 and 5.2% of their values in acetonitrile (Figure 49). The luminescence from 3 is approximately 20 fold greater in the presence of P450 than in buffer alone.

20 The 20-fold increase in integrated luminescence observed when 3 binds to P450 is favorable. However, the luminescence itself is weak in absolute terms. The reported quantum yield for $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ in aerated acetonitrile is 0.0073 (0.033 in deaerated acetonitrile). (Nair, R. B. et al., *Inorg. Chem.* (1997) 36, 962-965). Based on this measurement, the observed quantum yield of 3 bound to P450 would then be 0.00038.

25 There is some overlap between the Q bands of the P450 UV-visible absorption spectrum and the emission of the ruthenium complexes. However, a rough comparison with the known extent of Förster quenching of $\text{Ru}(\text{bpy})_3^{2+}$ complexes similarly bound to P450 suggests that energy transfer should be at most responsible for a 2-fold decrease in luminescence intensity. A more likely source of the low quantum yield is quenching by water. The model of 3 bound to P450 shows that the dppz phenazine nitrogens are still solvent-exposed (Figure 48). Although this model does not include the conformational

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changes the protein may make to accommodate the $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ complex, it illustrates the wide, solvent exposed channel that can be reasonably anticipated.

The concept of a molecular probe that emits light upon binding to its target enzyme has been successfully demonstrated to be conceptually sound. Detection may be improved in several ways. Luminescent probes can be designed to provide partial protection of the dppa ligand from solvent in order to achieve higher luminescence quantum yields in the presence of P450. For instance, the alkyl chain of the hypothetical molecule shown in Figure 50 should help exclude water from around the dppa ligand. Optical detection strategies can also be applied in the luminescent NOS probes.

EXAMPLE VII

This example describes luminescent sensitizer-linked substrate molecules as probes useful for detection of NOS for both in vivo imaging and drug design, a luminescence-based screen for NOS inhibitor affinity and isozyme specificity

NOS are involved in a plethora of both normal and pathological processes. Because of their biological and medicinal importance, it is crucial to develop modulators of activity, such as inhibitors for the NOS isozymes. The methods of the invention include a fluorescence-based screening technique lends itself to screening combinatorial libraries of NOS inhibitors. This assay is rapid, extremely sensitive, and provides accurate binding constants for the inhibitors being tested. The method can be applied for other heme enzymes, or any enzyme which absorbs light and for which an inhibitor or substrate is known.

The luminescent probes used for assessing the binding of isozyme specific inhibitors to NOS and for imaging the spatial distribution of NOS in living tissues are synthesized. Specifically, luminescent probes to be used for screening potential NOS inhibitors for binding are described.

Because the three NOS are involved in many different processes, it is desirable to inhibit only one isozyme at a time. This principle applies both to the molecular biology experiments necessary to elucidate the role of NOS isozymes in complex systems, and to the development of drugs to combat specific diseases. In order to achieve this goal a rapid, inexpensive, and sensitive screen must be developed to assay compounds for efficient and specific NOS inhibition. The luminescence assay of the invention meets all of these criteria.

Sensitizer-linked substrate molecules that bind competitively to the active site of NOS are synthesized. If the emission spectrum of the probe overlaps with the absorption of the heme, Förster energy transfer will quench virtually all of the probe's luminescence. However, if the probe is freed from NOS by competition with another inhibitor, luminescence will be restored. In order to demonstrate the promise of this technique, a brief discussion of the Förster theory is necessary.

The phenomenon of energy transfer between chromophores is well established, and has already found several uses in biotechnology and biophysics. (Wu, P.; Brand, L. *Analytical Biochemistry* (1994) 218, 1-13; Forster, T. in *Modern Quantum Chemistry* O. Sinanoglu, Ed. (Academic Press, New York, 1965), vol. 3, pp. 93-137) Briefly, the interaction between the energy donor and acceptor is modeled as the interaction of two dipoles, and thus falls off as r^6 . The rate of energy transfer between donor and acceptor is characterized by a parameter R_0 , which in turn depends on the overlap of the donor's fluorescence spectrum and the acceptor's absorption spectrum J :

$$R_0^6 = 8.8 \cdot 10^{-5} (\kappa^2 n^{-4} \phi_0 J)$$

$$J = \frac{\int_0^\infty F_D(\lambda) E_A(\lambda) \lambda^4 d\lambda}{\int_0^\infty F_D(\lambda) d\lambda}$$

R_0 also depends on ϕ_0 , the quantum yield in the absence of energy transfer, n , the index of refraction, and κ , an orientation factor dependent on the alignment of the donor and

acceptor dipoles. If the orientation between the donor and acceptor is random, $\kappa^2 = 2/3$.
The rate of energy transfer k_{en} is:

$$k_{en} = k_0 \left(\frac{R_0}{r} \right)^6$$

Here k_0 is the intrinsic rate of decay of the donor, and includes the rate of luminescent decay (k_{lum}) and the rate of non-radiative decay. Thus, a large R_0 or a short distance between donor and acceptor leads to fast energy transfer. In the absence of other decay paths, the decay rate of the donor in the presence of the acceptor will be:

$$k_{obs} = k_0 + k_{en}$$

The ratio of quantum yields of luminescence in the presence and absence of the acceptor is:

$$\begin{aligned} \frac{\phi_{obs}}{\phi_0} &= \frac{k_{lum}}{k_0 + k_{en}} \cdot \frac{k_0}{k_{lum}} \\ &= \frac{k_0}{k_0 + k_{en}} \\ &= \frac{1}{1 + \left(\frac{R_0}{r} \right)^6} \end{aligned}$$

Physically, the luminescent decay of the donor, which constitutes part of the decay rate k_0 , competes with the rate of energy transfer. In order to maximize the observed luminescence quenching, R_0 should be large (good overlap between donor and acceptor) and r should be small.

The sensitizer-linked substrate molecules developed herein have several practical advantages. The Soret bands of thiolate-ligated hemes have extinction coefficients of ca. 100,000 $\text{cm}^{-1}\text{M}^{-1}$. Provided that the emission spectrum of the sensitizer (i.e. fluorophore) falls in the vicinity of 400-450 nm, the overlap J will be large, and the quenching by energy transfer very efficient. Second, it is not necessary to develop a probe that is itself an excellent NOS inhibitor. In fact, the probe should bind with only moderate affinity so that a superior inhibitor will efficiently displace it. Third, the onset of luminescence can

be detected even at very low signal intensities. Fourth, screening for luminescence lends itself to high throughput screening techniques because of the possibility of examining many sample wells simultaneously. Fifth, the binding constant of the competing inhibitor will be easily determined based on the observed quantum yield in the presence of the competitor. (Dmochowski, I. J. et al., *Proc. Natl. Acad. Sci. USA* (1999) **96**, 12987-12990) Sixth, the general method described above should be applicable to any enzyme that absorbs light, provided that a suitable luminescent partner can be found.

In one embodiment of the invention, coumarins are used as the chromophores (Figure 51) (R is a known, NOS inhibitor). This choice was made for several reasons. First, various derivatives are available that fluoresce over a wide range of wavelengths. In particular, fluorophores A and B emit at about 446 and 410 nm, which provides good overlap with the heme Soret bands in both the high (~390 nm) and low (~420 nm) spin Fe^{III} states. (Leung, W.-Y. et al., *Bioorg. Med. Chem. Lett.* (1999) **9**, 2229-2232; Takadate, A. et al., *Chem. Pharm. Bull.* (1989) **37**, 373-376) Second, they both have high quantum yields: around 95% and 55% respectively. (Takadate, A. et al., *Chem. Pharm. Bull.* (1989) **37**, 373-376; Arbeloa, T. L. et al., *J. Phys. Chem.* (1993) **97**, 4704-4707) Third, they are small, and can therefore be placed close to the heme. Fourth, precursors are synthetically tractable and are commercially available (Sigma-Aldrich, St. Louis, MO, Molecular Probes, Eugene, OR). The R₀ of A with low spin Fe^{III} heme should be between 45 and 50 Å based on the overlap between the P450 water-bound Fe^{III} spectrum and the emission spectrum of A. Assuming a distance of 15 Å between A and the heme, the fluorescence quantum yield would decrease from about 95% to 0.1% upon binding to NOS.

Several classes of NOS inhibitors have been described in the literature. However, the dipeptide inhibitors based on N^ω-nitro-arginine (NMA) are well suited for this example (Huang, H. et al., *J. Med. Chem.* (1999) **42**, 3147-3153). The molecule NMA by itself is a good, but non-specific inhibitor of NOS. Fluorophores A and B are attached to the inhibitor directly, or through a tether if A and B prove to be too large to fit inside NOS (Figure 52). The length of the linker between the fluorophore and the NOS inhibitor can be varied from n = 1 to n = 10. Further, the linker may be replaced with an amino acid to

vary binding affinity and specificity. The syntheses are based on standard peptide coupling reactions.

The detection method of the invention (for NOS luminescent probes) is not dependent on either a specific fluorophore or NOS inhibitor. Other fluorophores are also applicable to this technique, including, but are not limited to, 2-amino-benzoic acids, Texas red, 1-and 2-aminonaphthalenes, p,p'-diaminostilbenes, pyrenes, anthracenes, fluoresceins, rhodamines, and other generally known luminescent dyes.

Substrates of NOS that can be used in the compositions and methods of the invention, include, but are not limited to, NOS inhibitors, such as N^G-monomethyl, dimethyl, nitro, and amino arginines, N^G-nitro-L-arginine methyl ester, N^δ-(iminoethyl-L-ornithine, L-thiocitrulline, S-alkyl-L-thiocitrulines, bithioureas, 7-nitroindazoles, aminogaunidine, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine, 2-iminoazaheterocycles, N-phenylisothioureas, and N-Phenylamidines. (Collins, J.L. et al., *J. Med. Chem.* (1998) 41, 2858-2871; Hibbs, J.B. et al. *J. Immunol.* (1987) 138, 550-565; Lamber, L.E. et al., *Life Sci.* (1991) 48, 69-75; Rees, D.D. et al. *Br. J. Pharmacol.* (1990) 101, 746-752; Gross, S.S. et al., *Biochem. Biophys. Res. Commun.* (1990) 270, 96-203; Furfine, E.S. et al., *Biochemistry* (1993) 32, 8512-8517; Narayanan, K. et al., *J. Med. Chem.* (1994) 37, 885-887; Narayanan, K. et al., *J. Biol. Chem.* (1995) 270, 11103-11110; Furfine, E.S. et al., *J. Biol. Chem.* (1994) 269, 26677-26683; Garvey, E.P. et al., *J. Biol. Chem.*, (1994) 269, 26669-26676; Wolff, D.J. et al., *Arch. Biochem. Biophys.* (1994) 311, 300-306; Hasan, K. J. et al., *Pharmacol.* (1993) 249, 101-106; Moore, W.M. et al., *J. Med. Chem.* (1996) 39, 669-672; Moore, W.M. et al., *Bioorg. Med. Chem.* (1996) 4, 1559-1564; Shearer, B.G. et al., *J. Med. Chem.*, (1997) 40, 1901-1905; Garvey, E.P. et al., *J. Biol. Chem.* (1997) 272, 4959-4963. Cowart, M. et al., *J. Med. Chem.* (1998) 41, 2636-2642)

EXAMPLE VIII

This example describes luminescent probes for imaging the spatial distribution of NOS in vivo.

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This example describes a class of sensitizer-linked substrate molecules (i.e. probe molecules) that begin to luminesce upon binding to NOS. The probes are designed to be specific to NOS isozymes. These sensitizer-linked substrate molecules offer researchers a new tool for studying the localization of NOS isozymes *in vivo*. Unlike many current techniques, this method is non-destructive. Because the probe molecules are reversible inhibitors, the probes should leave NOS function intact. Further, it offers the sensitivity inherent in luminescence assays. The Ruthenium *tris*-bipyridine (Ru(bpy)₃) excitation (450 nm) and luminescence (620 nm) are far to the red of the excitation and emission spectra of biological molecules, and thus avoid the difficulties of background fluorescence from the sample. The general design of a Ru(bpy)₃ moiety tethered to a substrate that functions as a luminescence quencher is applicable in a wide variety of systems.

The Ruthenium *tris*-bipyridine based luminescent probes are designed such that upon excitation with light of around 450 nm, an electron transfers from the Ru^{II} center to one of the bipyridine ligands (Figure 53)(Horvath, O.; Stevenson, K. L. *Charge Transfer Photochemistry of Coordination Compounds* (VCH Publishers, Inc., New York, NY, 1993)). Quenching does not necessarily lead to charge-separated products. Depending on the quencher, geminate recombination may be much faster than solvent cage escape. This excited state undergoes luminescent decay back to the ground state with a quantum yield of about 4.2%. The luminescence spectrum is centered around 620 nm, far to the red of the background fluorescence of biological samples. The excited state is both an excellent reductant and oxidant, and in the presence of a suitable redox partner can form either [Ru(bpy)₃]³⁺ or [Ru(bpy)₂(bpy^{•+})]⁺. The former process is called oxidative quenching, while the latter process is termed reductive quenching. When the excited state is intercepted by a quencher it does not luminesce. Quenching does not necessarily lead to

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charge-separated products. Depending on the quencher, geminate recombination may be much faster than solvent cage escape.

The synthetic strategy of luminescent probes of the invention is shown in Figure 54.

- 5 Nitrobenzene quenches the Ru^{2+} excited state through electron transfer. These probes should emit very little light when free in solution, but luminesce brightly upon binding to NOS. Two variations of probe molecules are shown. Class C is comprised of arginine mimics connected to nitrophenylalanine, which is in turn connected to $[\text{Ru}(\text{bpy})_3]^{2+}$ though a long tether. A similar inhibitor, D-Phe-D-ArgNO₂-OMe binds n and eNOS with
10 micromolar dissociation constants, but binds iNOS with a 3.6 mM K_d . (Huang, H. et al., *J. Med. Chem.* (1999) 42, 3147-3153) The probes in class D are based on the inhibitor 1400W, which is an irreversible inhibitor of iNOS, but a weak, reversible inhibitor of nNOS and eNOS. (Garvey, E. P. et al., *J. Biol. Chem.* (1997) 272, 4959-4963).
- 15 The $[\text{Ru}(\text{bpy})_3]^{2+}$ excited state is efficiently quenched by nitrobenzene through electron transfer. (Meyerstein, D. et al., *J. Phys. Chem.* (1978) 82, 1879-1885) However, the reverse electron transfer is extremely rapid, so no net charge separation occurs. In solution, the flexible linkers allow the quencher to fold back upon the ruthenium. Close proximity should lead to efficient quenching of the $[\text{Ru}(\text{bpy})_3]^{2+}$ luminescence. In
20 contrast, when the probe is bound to NOS the quencher is sequestered within the enzyme. Because the rate of electron transfer through saturated bonds decreases exponentially with distance, the ruthenium luminescence is no longer be quenched.

The through-bond model for electron transfer validates the detection system described.

- 25 For a given path between electron donor and acceptor, the rate of electron transfer is predicted to depend on the number of intervening covalent bonds N_C , the number of hydrogen bonds N_H , the number of through space contacts N_S , and the total length of through space jumps R_{space} : (Beratan, D. N.; Skourtis, S. S. *Curr. Opin. Chem. Biol.* (1998) 2, 235-243).

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Although more rigorous models exist, this one illustrates several important points. First, the rate of electron transfer through covalent, saturated bonds decreases exponentially with the number of bonds. Second, the rate of electron transfer through a van der Waals contact is roughly the same as through a covalent bond. Third, electron transfer through space is possible over short distances (1-3 Å), but very unfavorable over long distances. In solution, the nitrobenzene and ruthenium are able to make direct contact, so electron transfer should be fast. However, when the nitrobenzene moiety is bound inside the enzyme the electron will be forced to tunnel along the tether. If the tether connecting the ruthenium to the nitrobenzene moiety is 10 carbons long (11 bonds) the rate of electron transfer should be approximately $4 \times 10^5 \text{ sec}^{-1}$ slower than electron transfer through one covalent contact. This corresponds to a predicted electron transfer rate of $1.4 \cdot 10^5 \text{ sec}^{-1}$, or about one-tenth the normal rate of decay of photoexcited $[\text{Ru}(\text{bpy})_3]^{2+}$.

Researchers interested in the rate of electron transfer through flexible hydrocarbon chains have observed that the rate of electron transfer became independent of chain length when the chain became more than 6 carbons long, with $k \approx 4 \cdot 10^7 \text{ sec}^{-1}$ (Figure 55). (Yonemoto, E. H. et al., *J. Am. Chem. Soc.* (1994) **116**, 4786-4795). Methyl viologen is an efficient oxidative quencher. This rate of quenching is 20 times faster than the natural rate of decay of $[\text{Ru}(\text{bpy})_3]^{2+}$ (about $2 \cdot 10^6 \text{ sec}^{-1}$), and would decrease the luminescence quantum yield to about 5% of the normal $\text{Ru}(\text{bpy})_3$ quantum yield. Because the methyl viologen quencher used by these researchers and nitrobenzene have similar bimolecular quenching rate constants, a nitrobenzene moiety connected to $[\text{Ru}(\text{bpy})_3]^{2+}$ by an alkyl chain at least 6 carbons long should exhibit a similar rate of quenching when free in solution. (Meyerstein, D. et al., *J. Phys. Chem.* (1978) **82**, 1879-1885; Hoffman, M. Z. et al., *J. Phys. Chem. Ref. Data* (1989) **18**, 219-543).

Yonemoto *et al.* measured the rate of electron transfer between $\text{Ru}(\text{bpy})_3$ and methyl viologen connected by an eight-carbon tether in the presence and absence of a β -cyclodextrin. When the alkyl chain was threaded through the β -cyclodextrin, the rate of electron transfer dropped from 2.4×10^7 to $1.8 \times 10^5 \text{ sec}^{-1}$, which is again one tenth of the natural decay rate of $\text{Ru}^{\text{II}}(\text{bpy})_3$. (Yonemoto, E. H. et al., *J. Am. Chem. Soc.* (1994) **116**,

4786-4795) Thus, both previous research and order of magnitude calculations illustrate that the ruthenium luminescence should be efficiently quenched while the probe is free in solution, but restored when the nitrophenyl group is sequestered inside NOS, suggesting that such a probe would be optimal in the methods of the invention.

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The syntheses of these inhibitors are based on standard peptide coupling reactions. Carboxylate terminated $[\text{Ru}(\text{bpy})_3]^{2+}$ tethers have been synthesized previously(Wilker, J. J. et al., *Angew. Chem. Int. Ed. Eng.* (1999) **38**, 90-92). The stereochemistry, length of the tether to $\text{Ru}(\text{bpy})_3$, and arginine mimic can all be varied to optimize binding and selectivity. Both the inhibitors to be used and the nitrophenylalanine are commercially available. The syntheses of the class **D** probes are outlined in Figure 56. 1400W is identical to 15 with the exception of the addition of the nitro group. The synthesis of the amidine functional group (conversion of **14** to **D**) follows the preparation reported by the discoverers of 1400W and related inhibitors.(Collins, J. L. et al., *J. Med. Chem.* (1998) **41**, 2858-2871). 1400W and related inhibitors exhibit outstanding binding and selectivity and may make particularly advantageous probes.

The modular design of these probes makes them easy to test during their development. The bimolecular quenching rate constant of $[\text{Ru}(\text{bpy})_3]^{2+}$ can be measured by nitrophenylalanine or by **12** and **15**. Similarly, **15** and **13** are tested for binding affinity to NOS before being incorporated into the final probe molecules. In addition to the described probes, other probes may be made based on the wide variety of luminescent metal complexes and NOS inhibitors that are available.(Hoffman, M. Z. et al., *J. Phys. Chem. Ref. Data* (1989) **18**, 219-543; Furfine, E. S. et al., *J. Biol. Chem.* (1994) **269**, 2667-26683; Garvey, E. P. et al., *J. Biol. Chem.* (1994) **269**, 26669-26676; Shearer, B. G. et al., *J. Med. Chem.* (1997) **40**, 1901-1905), and references therein.

Other inhibitors of NOS include, but are not limited to, N^G -monomethyl, dimethyl, nitro, and amino arginines, N^G -nitro-L-arginine methyl ester, N^δ -(iminoethyl-L-ornithine, L-thiocitrulline, S-alkyl-L-thiocitrulines, bistioureas, 7-nitroindazoles, aminogaunidine, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine, 2-iminoazaheterocycles, N-

- phenylisothioureas, N-phenylamidines and modifications of these compounds.(Collins, J.L. et al., *J. Med. Chem.* (1998) **41**, 2858-2871; Hibbs, J.B. et al. *J. Immunol.* (1987) **138**, 550-565; Lamber, L.E. et al., *Life Sci.* (1991) **48**, 69-75; Rees, D.D. et al. *Br. J. Pharmacol.* (1990) **101**, 746-752; Gross, S.S. et al., *Biochem. Biophys. Res. Commun.* (1990) **270**, 96-203; Furfine, E.S. et al., *Biochemistry* (1993) **32**, 8512-8517; Narayanan, K. et al., *J. Med. Chem.* (1994) **37**, 885-887; Narayanan, K. et al., *J. Biol. Chem.* (1995) **270**, 11103-11110; Furfine, E.S. et al., *J. Biol. Chem.* (1994) **269**, 26677-26683; Garvey, E.P. et al., *J. Biol. Chem.*, (1994) **269**, 26669-26676; Wolff, D.J. et al., *Arch. Biochem. Biophys.* (1994) **311**, 300-306; Hasan, K. J. et al., *Pharmacol.* (1993) **249**, 101-106;
- 10 Moore, W.M. et al., *J. Med. Chem.* (1996) **39**, 669-672; Moore, W.M. et al., *Bioorg. Med. Chem.* (1996) **4**, 1559-1564; Shearer, B.G. et al., *J. Med. Chem.*, (1997) **40**, 1901-1905; Garvey, E.P. et al., *J. Biol. Chem.* (1997) **272**, 4959-4963. Cowart, M. et al., *J. Med. Chem.* (1998) **41**, 2636-2642).
- 15 Other luminescent metal complexes include, but are not limited to, homo- and heteroleptic ruthenium terpyridine. bipyridine, pyridine, imidazole, cyano and carbonyl complexes, as well as complexes of other transition metals, including but are not limited to osmium, platinum, iridium, rhenium, rhodium, molybdenum, tungsten and copper.[Roundhill, D.M. *Photochemistry and Photophysics of Metal Complexes* (Plenum
- 20 Press, New York, 1994); Horvath, O. and Stevenson, K.L. *Charge Transfer Photochemistry of Coordination Compounds* (VCH Publishers, Inc., New York, 1992)] Other luminescence quenchers include, but are not limited to, methyl viologens, quinones, N,N-dialkylanilines, N,N-dialkyl-p-methoxyanilines and triarylaminines. [Hoffman, M.Z. *J. Phys. Chem. Ref. Data* (1989) **18**, 219-543).

What is claimed:

1. A sensitizer-linked substrate molecule comprising a substrate and a sensitizer connected by a linker.
- 5 2. The molecule of claim 1, wherein said substrate is a binding element of a target biomolecule.
- 10 3. The molecule of claim 1, wherein said sensitizer is located within or near (< 100 Å from) the surface of a target biomolecule when the substrate of the sensitizer-linked substrate molecule is bound to the target biomolecule.
4. The molecule of claim 1, wherein said biomolecule is a metalloprotein.
- 15 5. The molecule of claim 4, wherein said metalloprotein is a heme protein.
6. The molecule of claim 4, wherein said biomolecule is Cytochrome P450.
7. The molecule of claim 1, wherein said sensitizer is a photosensitizer.
- 20 8. The molecule of claim 7, wherein said photosensitizer is a $\text{Ru}(\text{bpy})_3^{2+}$ complex.
9. The molecule of claim 8, wherein said $\text{Ru}(\text{bpy})_3^{2+}$ complex is the Δ or Λ enantiomer.
- 25 10. The molecule of claim 7, wherein said photosensitizer is selected from the group $[\text{Ru}(\text{phen})_2\text{dppz}]^{2+}$ or $[\text{Ru}(\text{phen})_2\text{dppa}]^{2+}$.
11. The molecule of claim 7, wherein said photosensitizer is a coumarin molecule.

12. The molecule of claim 1, wherein said linker is a molecule of sufficient length to allow the substrate to bind to the active site of the biomolecule so that upon binding the sensitizer is located at or near the surface of the biomolecule.

13. The molecule of claim 12, wherein said linker is an alkyl chain, $(CH_2)_n$, in which n is from 1 to 13.

14. The molecule of claim 1, wherein said substrate is a molecule that binds to Cytochrome P450.

15. The molecule of claim 14, wherein, said substrate selected from the group consisting of adamantane (Ad), ethylbenzene (EB), and imidazole (Im).

16. The molecule of claim 1, wherein said sensitizer-linked substrate molecule is selected from the group consisting of $[Ru-C_{13}-EB]^{2+}$, $[Ru-C_{12}-EB]^{2+}$, $[Ru-C_{11}-EB]^{2+}$, $[Ru-C_{10}-EB]^{2+}$, $[Ru-C_9-EB]^{2+}$, $[Ru-C_7-EB]^{2+}$, $[Ru-C_{11}-Ad]^{2+}$, $[Ru-C_9-Ad]^{2+}$, $[Ru-C_{11}-Im]^{2+}$, $[Ru-C_{13}-Im]^{2+}$, Ru-dppa- C_6 -Ad, Ru-dppa-gly-Ad, and Ru-dppa-Ad.

17. The molecule of claim 1, wherein said sensitizer-linked substrate molecule is selected from the group consisting of $\Delta-[Ru-C_9-Ad]^{2+}$ and $\Lambda-[Ru-C_9-Ad]^{2+}$.

18. The molecule of claim 1, wherein the said sensitizer-linked substrate molecule is selected from the group consisting of 4-, 6-, 7- substituted coumarin classes A and B, as shown in Figure 51.

19. The molecule of claim 1, wherein the said sensitizer-linked substrate molecule is selected from the group consisting of classes C and D, as shown in Figure 54.

20. A method of detecting a target biomolecule in a sample comprising contacting a target biomolecule with a sensitizer-linked substrate molecule that recognizes the target biomolecule so that a biomolecule/sensitizer-linked substrate complex is

32. The method of claim 20, wherein said linker is an alkyl chain, $(CH_2)_n$, wherein $n=1-13$.

5 33. The method of claim 20, wherein, said substrate is a molecule that binds to the active site of Cytochrome P350.

34. The method of claim 33, wherein the said substrate is selected from the group consisting of adamantane (Ad), ethylbenzene (EB), imidazole (Im).

10

35. The method of claim 26, wherein, the said sensitizer-linked substrate molecule is selected from the group consisting of 4-, 6-, 7- substituted coumarin classes A and B, as shown in Figure 51.

15 36. The method of claim 26, wherein, the said sensitizer-linked substrate molecule is selected from the group consisting of classes C and D, as shown in Figure 54.

37. A method for identifying an agent of interest that modulates a target biomolecule activity in a sample comprising:

20

a) contacting said target biomolecule and sensitizer-linked substrate molecule with a the agent of interest; and

b) detecting the signal emitted by the free sensitizer-linked substrate molecule and/or a signal resulting from a combination of the biomolecule and sensitizer-linked
25 substrate, the change in the signal being indicative that the agent of interest modulates the target biomolecule in the sample.

(19) World Intellectual Property Organization
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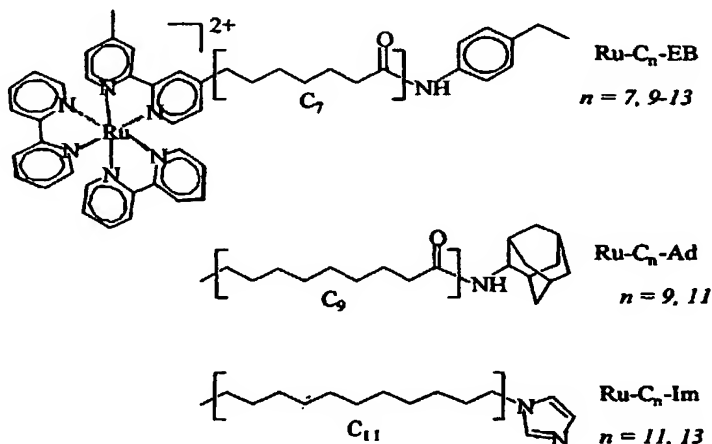
(43) International Publication Date
25 January 2001 (25.01.2001)

PCT

(10) International Publication Number
WO 01/06260 A1

- (51) International Patent Classification⁷: G01N 33/58, 33/542, C12Q 1/26
- (21) International Application Number: PCT/US00/19821
- (22) International Filing Date: 19 July 2000 (19.07.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/144,488 19 July 1999 (19.07.1999) US
60/149,278 16 August 1999 (16.08.1999) US
60/192,703 28 March 2000 (28.03.2000) US
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- (74) Agent: ADRIANO, Sarah, B.; Mandel & Adriano, Suite 60, 35 No. Arroyo Parkway, Pasadena, CA 91103 (US).
- (81) Designated States (national): AE, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:
— With international search report.
— Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: DETECTION OF BIOMOLECULES BY SENSITIZER-LINKED SUBSTRATES



(57) Abstract: Methods and compositions for detecting and characterizing target biomolecules using sensitizer-linked substrate molecules are disclosed. High throughput screening assays and therapeutic applications of the inventions are also included.

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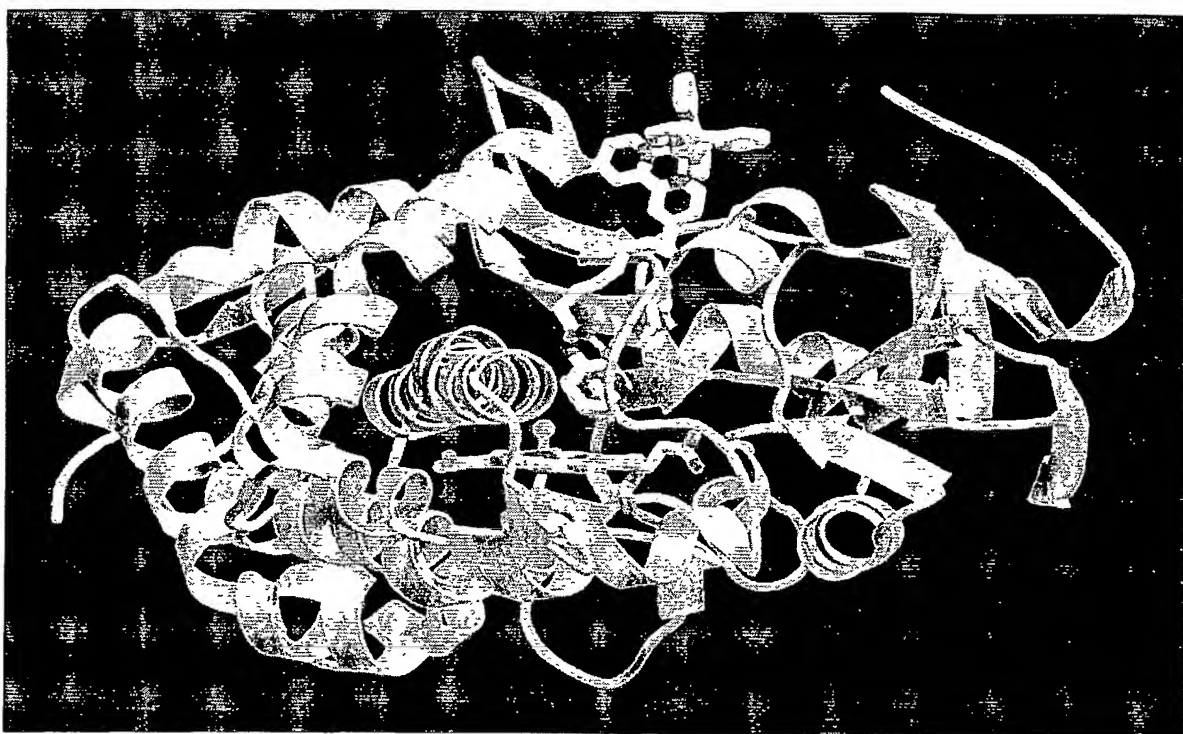


FIG. 1

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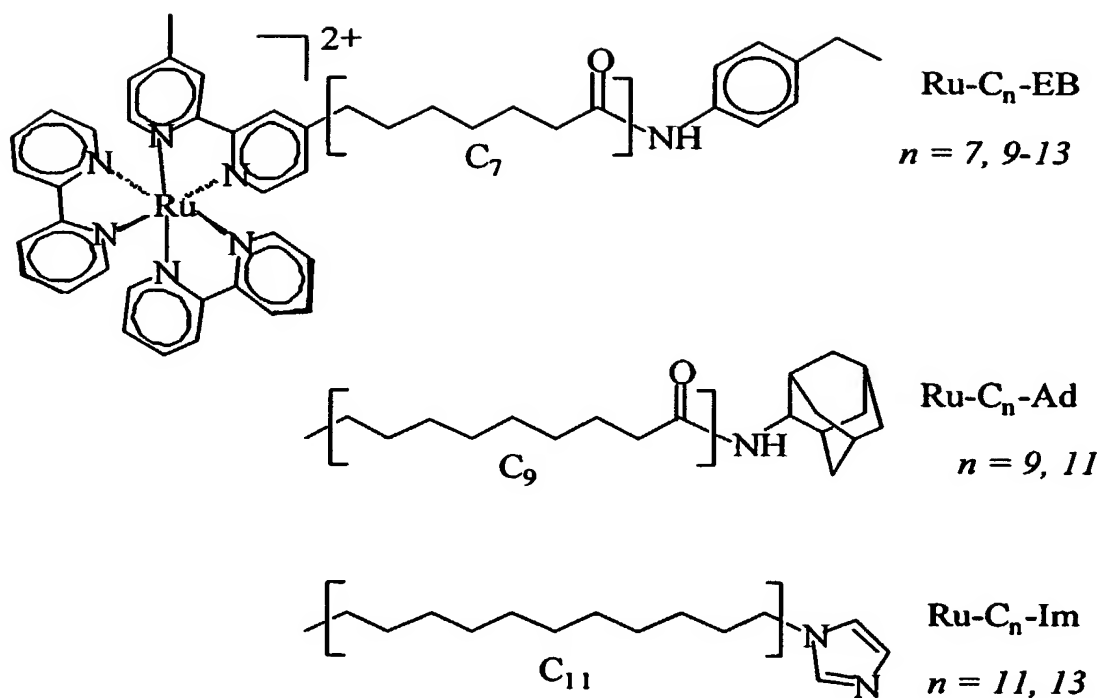


FIG. 2

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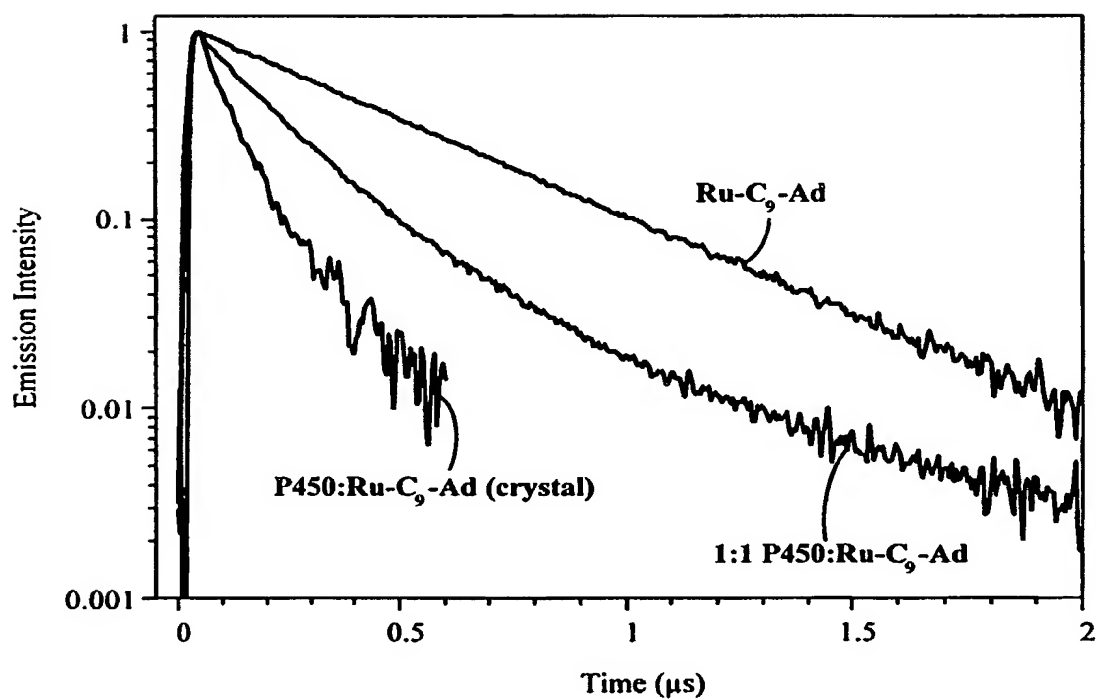


FIG. 3

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compound	$K_D(\mu\text{M})$	$k_{\text{on}}^{-1}(\text{ns})$	Ru-Fe(Å)
$[\text{Ru-C}_{13}\text{-EB}]^{2+}$	1.7 ± 0.4	107 ± 8	20.6 ± 0.2
$[\text{Ru-C}_{12}\text{-EB}]^{2+}$	1.5 ± 0.3	103 ± 7	20.5 ± 0.2
$[\text{Ru-C}_{11}\text{-EB}]^{2+}$	0.9 ± 0.4	94 ± 7	20.1 ± 0.3
$[\text{Ru-C}_{10}\text{-EB}]^{2+}$	0.9 ± 0.4	88 ± 2	19.9 ± 0.1
$[\text{Ru-C}_9\text{-EB}]^{2+}$	0.7 ± 0.1	75 ± 2	19.4 ± 0.1
$[\text{Ru-C}_7\text{-EB}]^{2+}$	6.5 ± 1.3	77 ± 2	19.5 ± 0.1
$[\text{Ru-C}_{11}\text{-Ad}]^{2+}$	0.8 ± 0.3	203 ± 16	21.0 ± 0.3
$[\text{Ru-C}_9\text{-Ad}]^{2+}$	0.7 ± 0.2	231 ± 11	21.4 ± 0.2
$[\text{Ru-C}_{13}\text{-Im}]^{2+}$	4.1 ± 1.1	190 ± 8	21.2 ± 0.1
$[\text{Ru-C}_{11}\text{-Im}]^{2+}$	-----	488 ± 35	N/A

Uncertainties represent one standard deviation of the data averaged from three to six experiments.

FIG. 4

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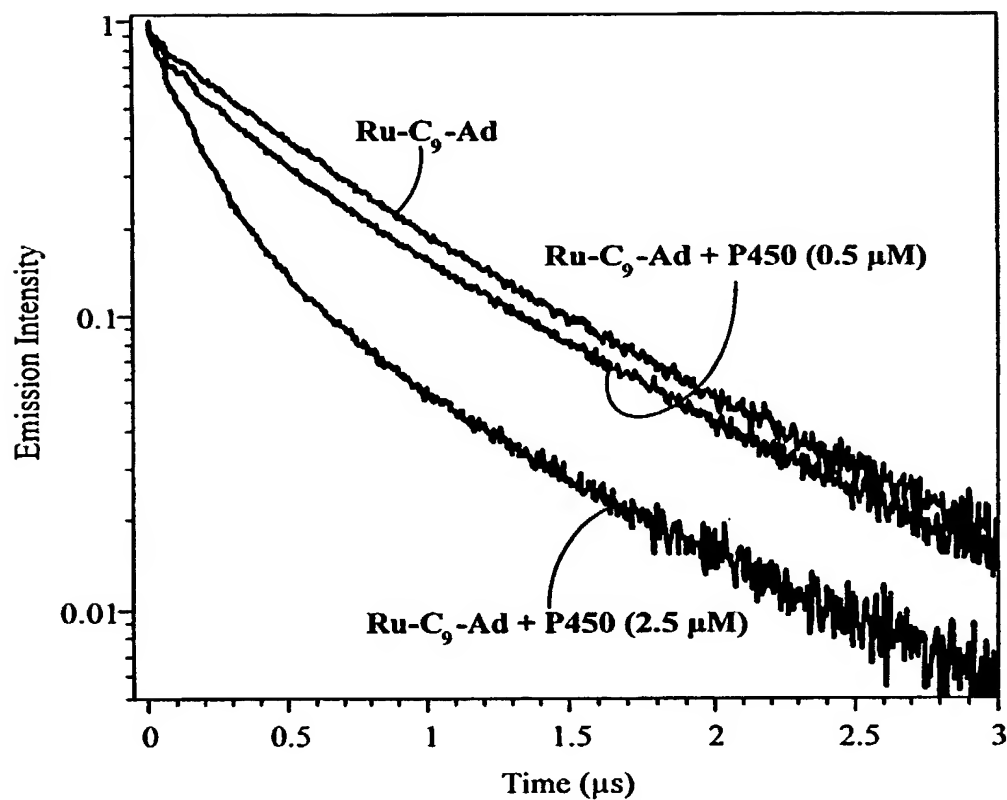


FIG. 5

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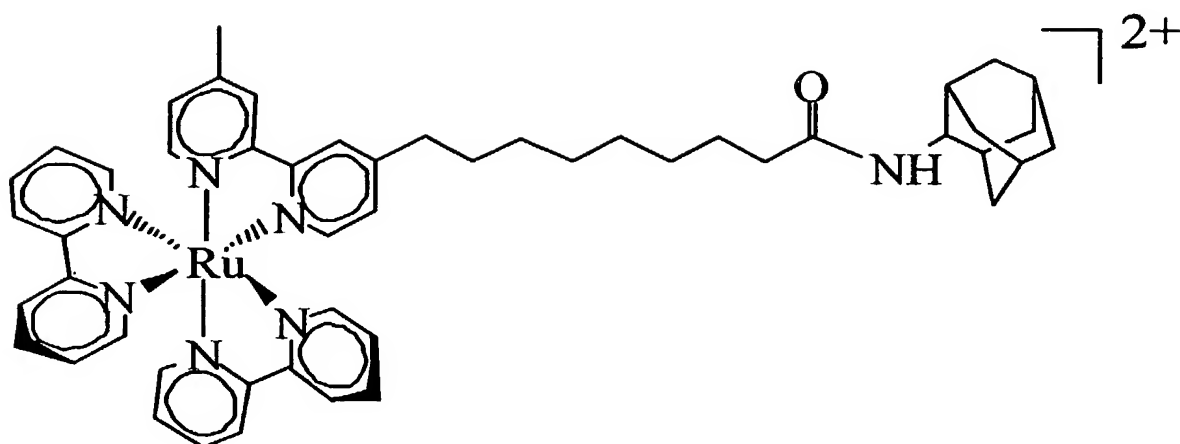


FIG. 6

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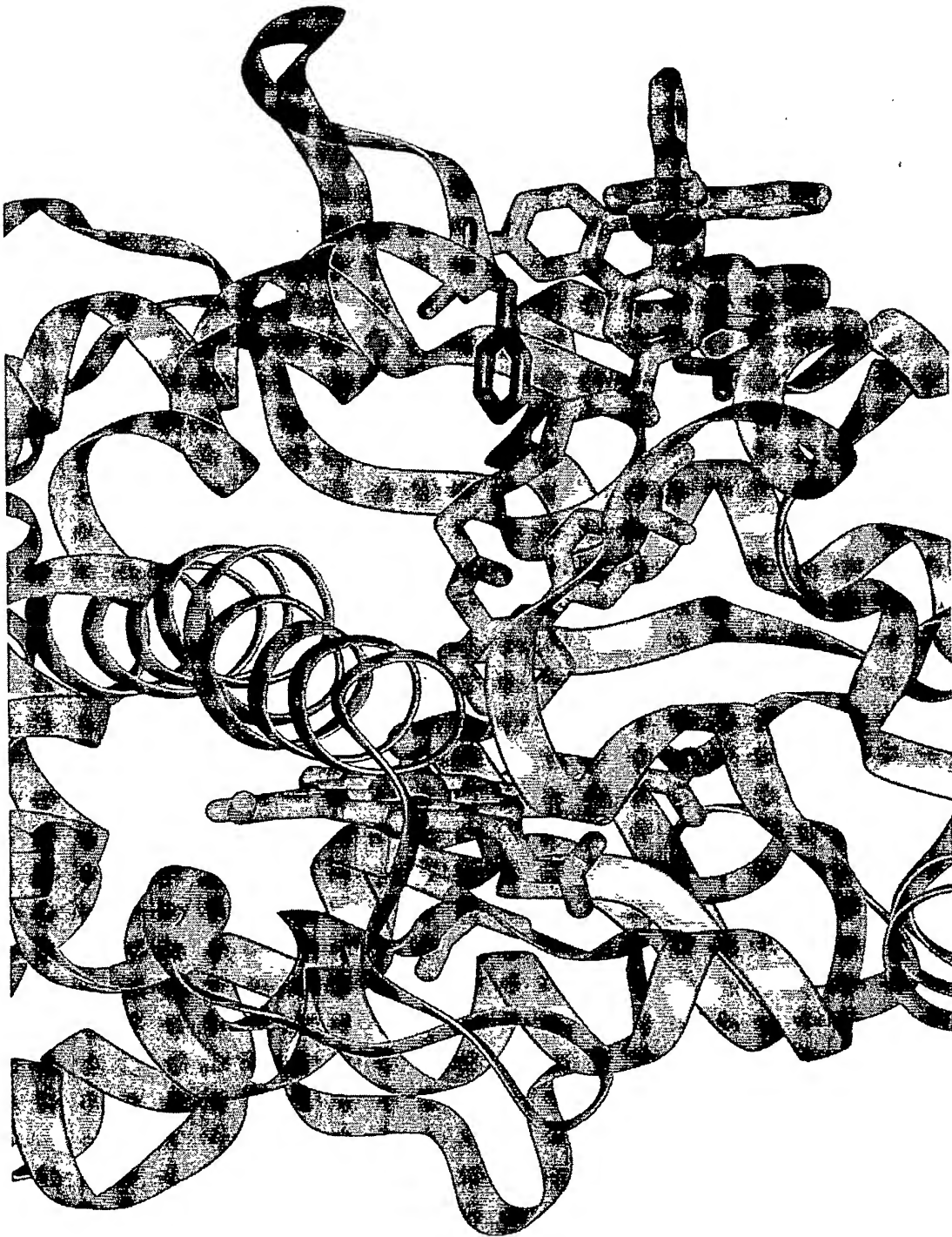


FIG. 7

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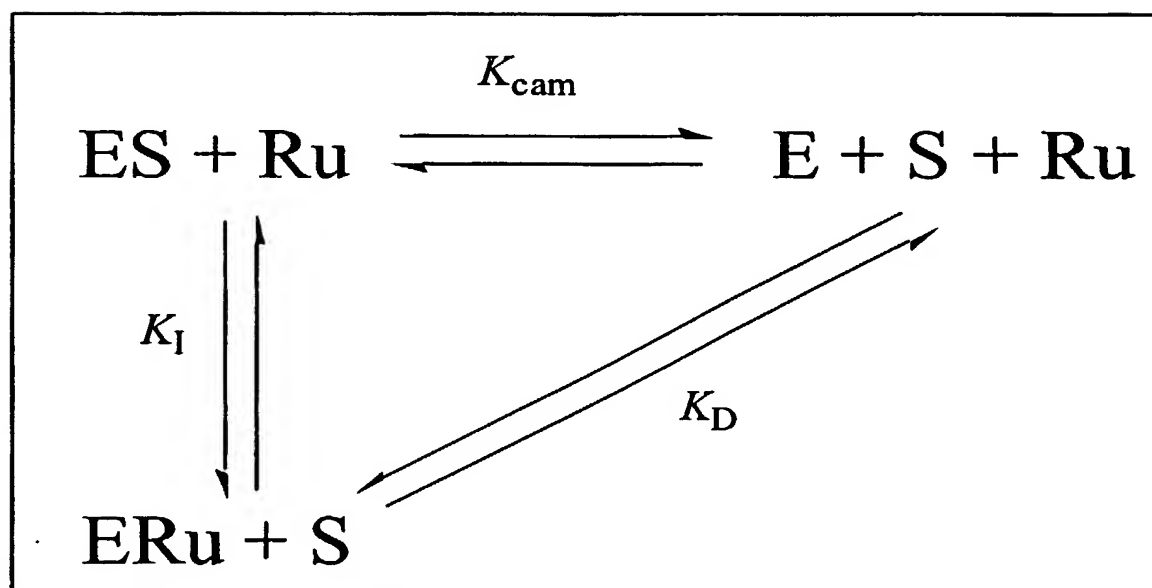


FIG. 8

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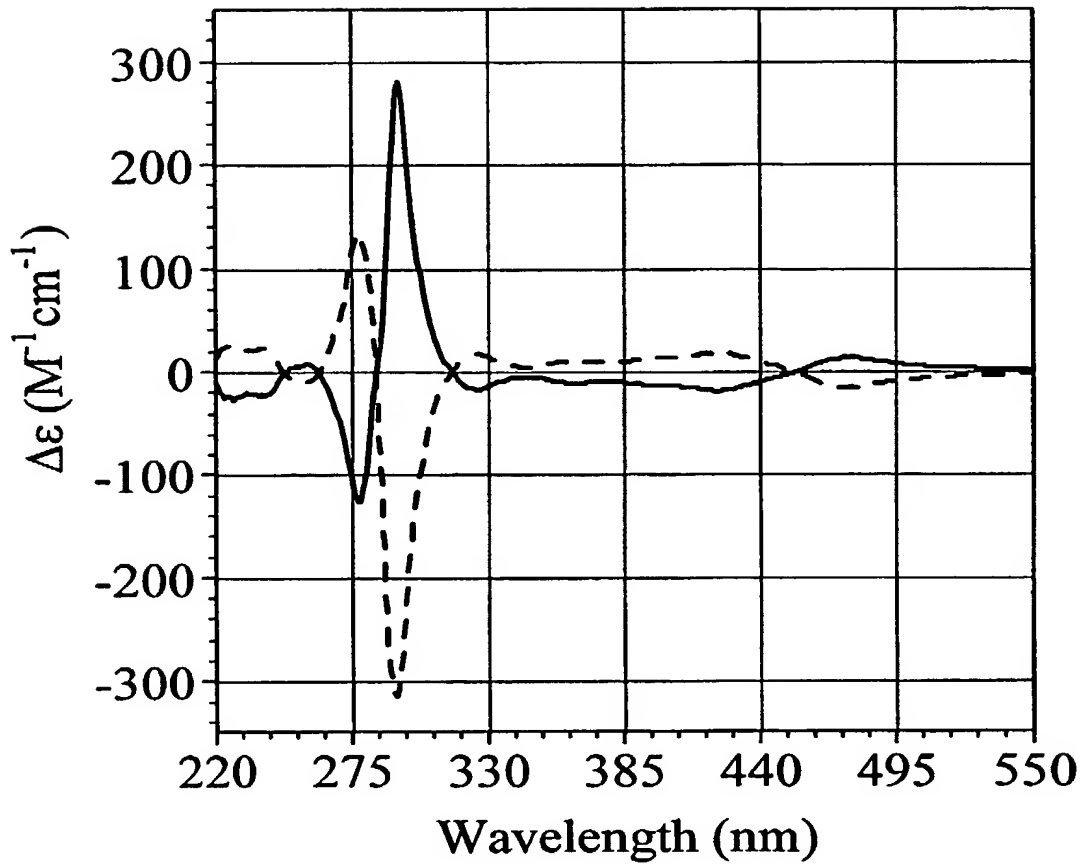


FIG. 9

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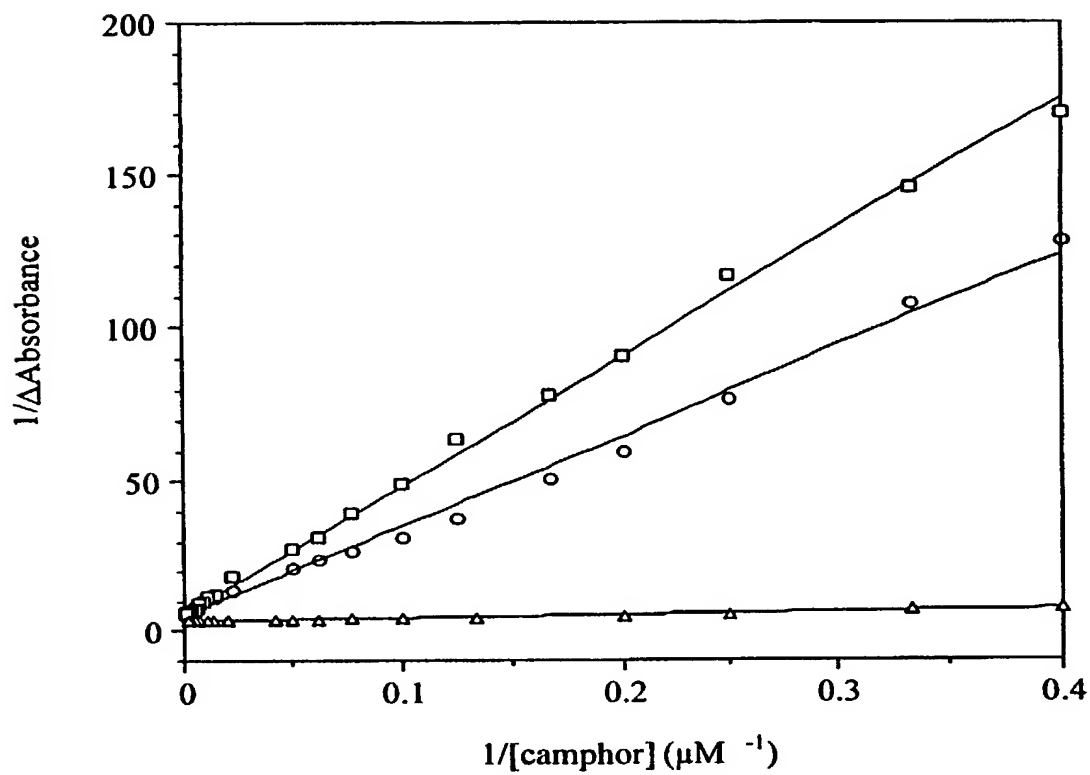


FIG. 10

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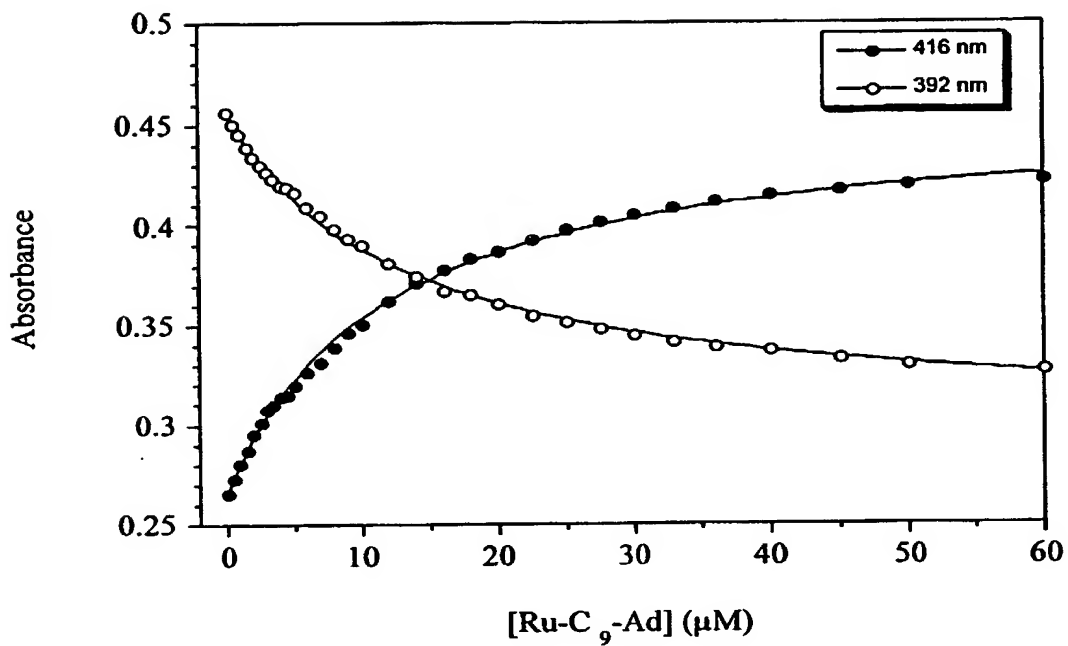


FIG. 11

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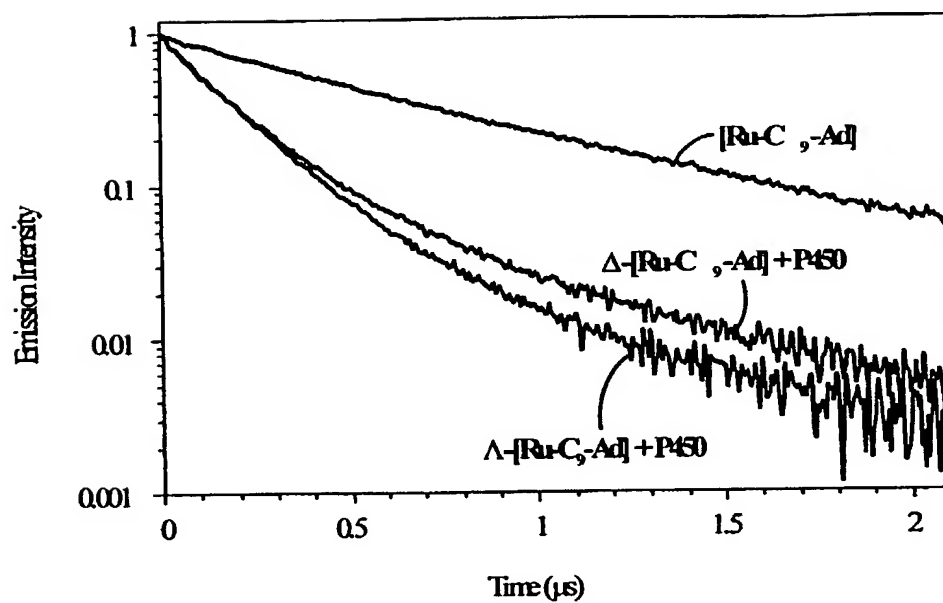


FIG. 12

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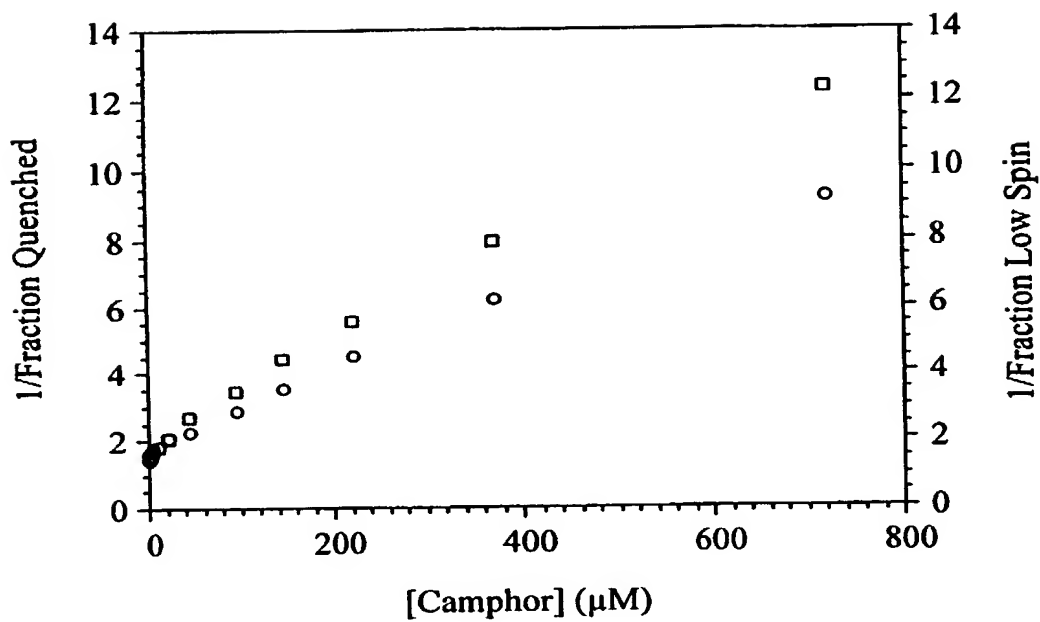
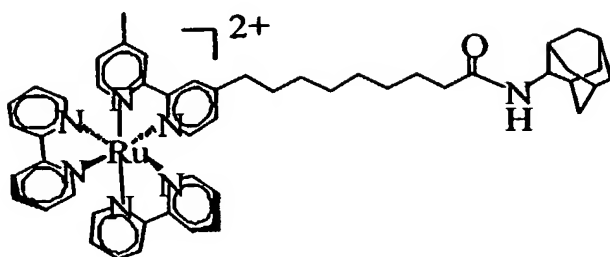


FIG. 13

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(1) $[\text{Ru-C}_9\text{-Ad}]^{2+}$ 

(2) 2-Adamantylacetamid

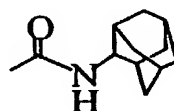
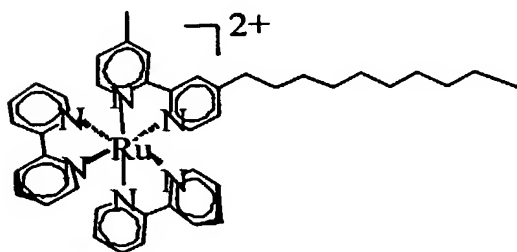
(3) $[\text{Ru-C}_{10}]^{2+}$ 

FIG. 14

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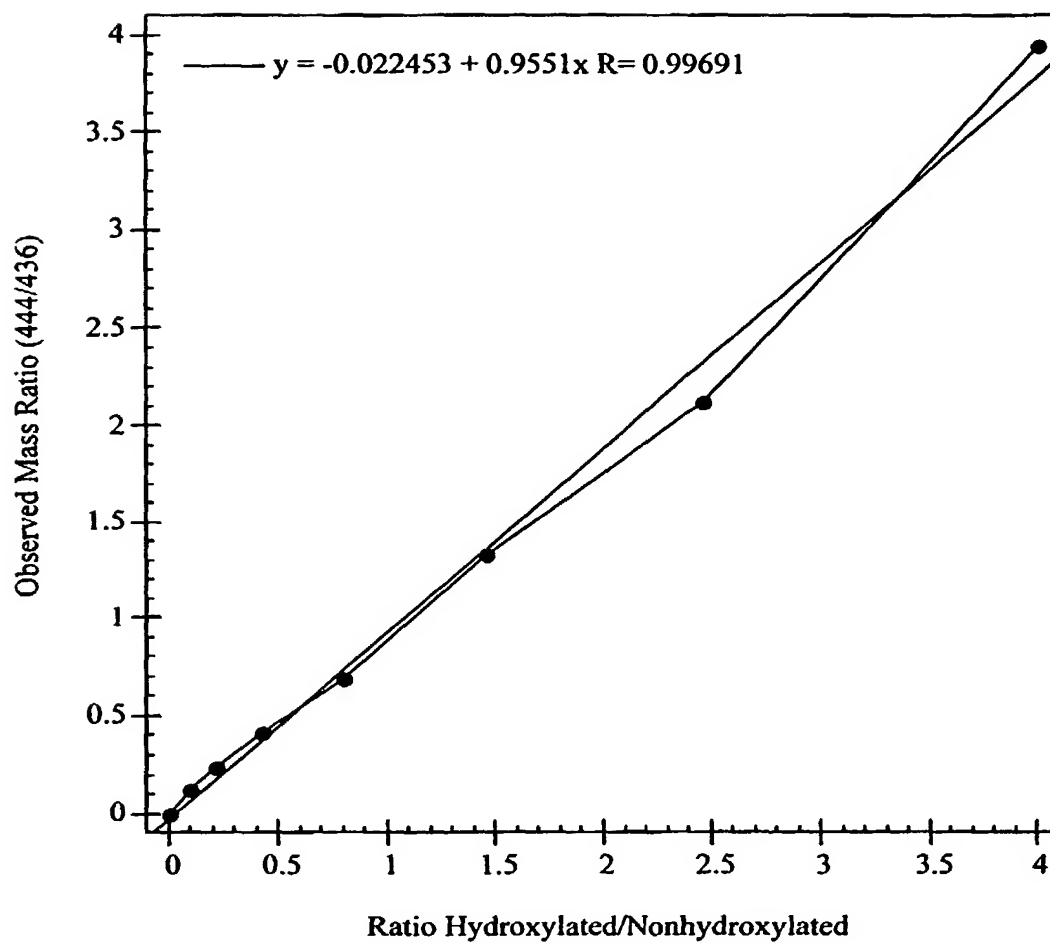


FIG. 16

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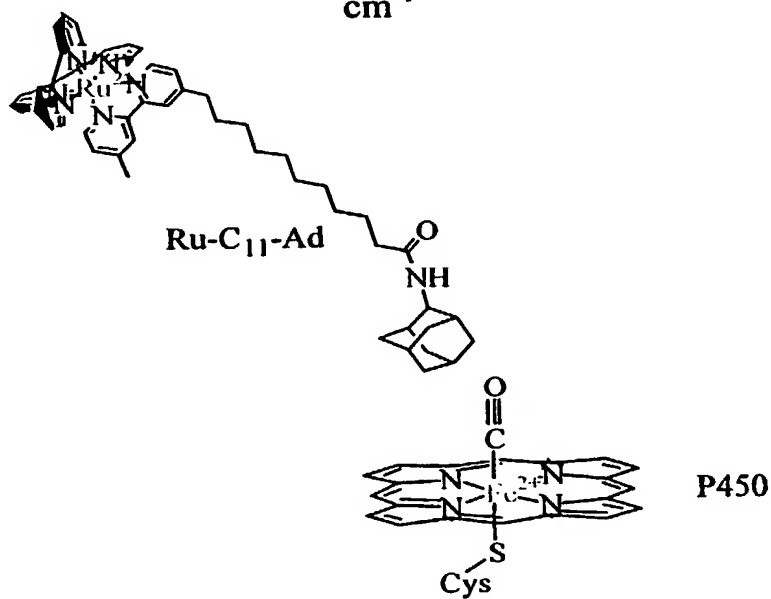
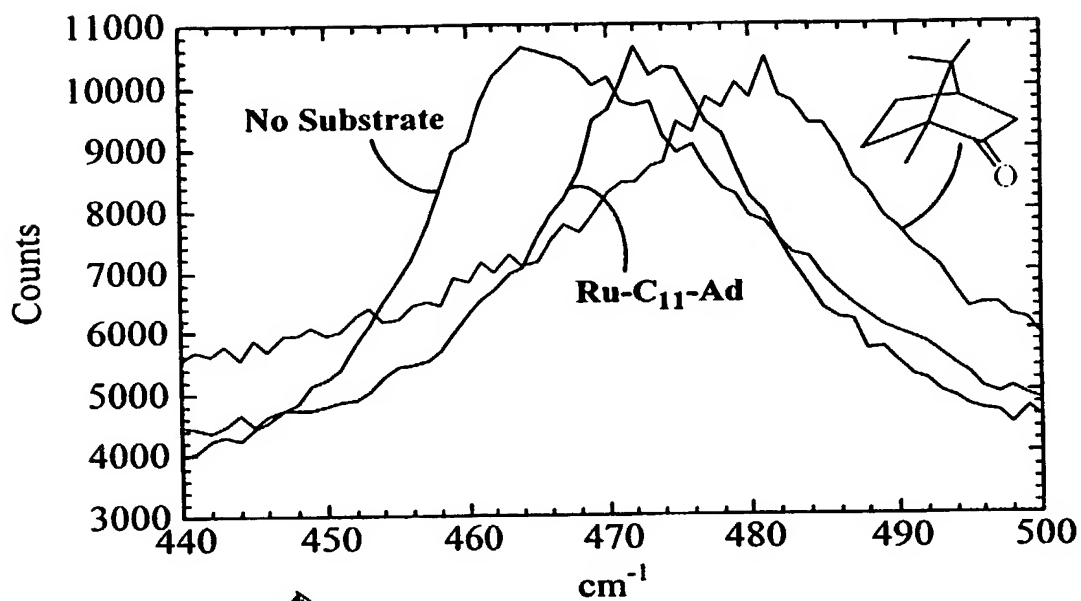


FIG. 17

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<u>Substrate</u>	<u>ν Fe-(CO)^a</u>
None	463 ^b
Ru-C ₁₁ -Ad	472
Ru-C ₉ -Ad	474
Adamantane	476
Camphor	482 ^b

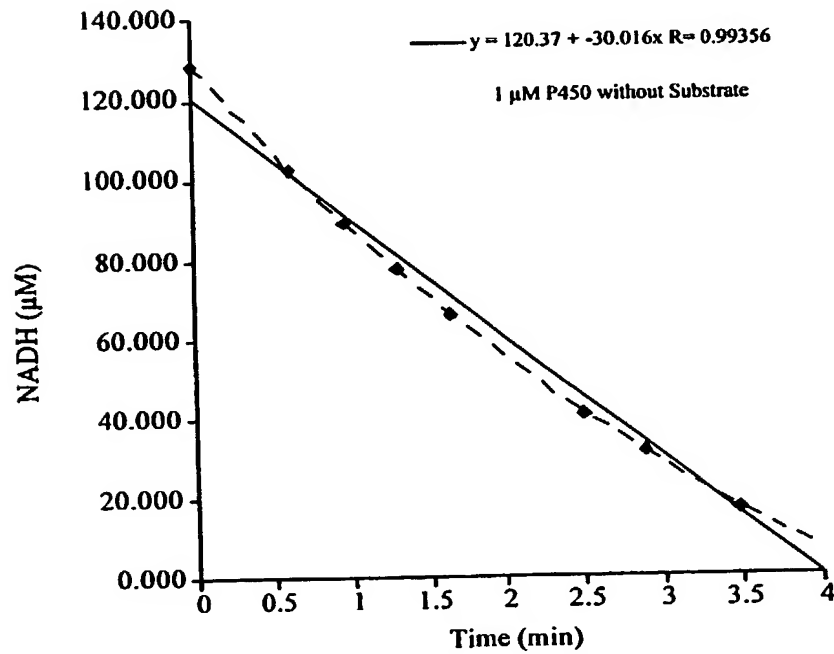
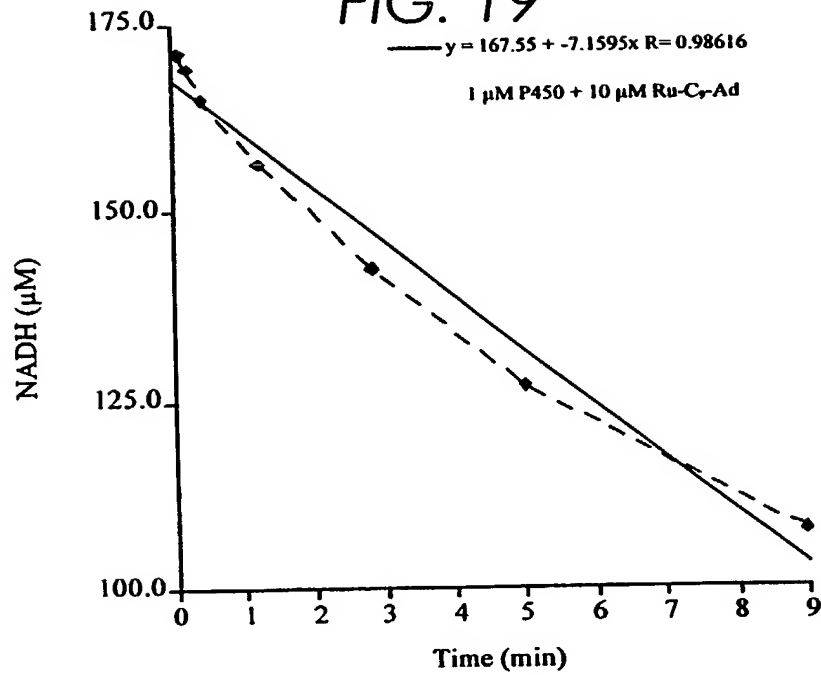
^aAll units are in cm⁻¹.

^bThese values are in good agreement with published values for substrate-free (465 cm⁻¹); and camphor-bound P450 (484 cm⁻¹) (19).

FIG. 18

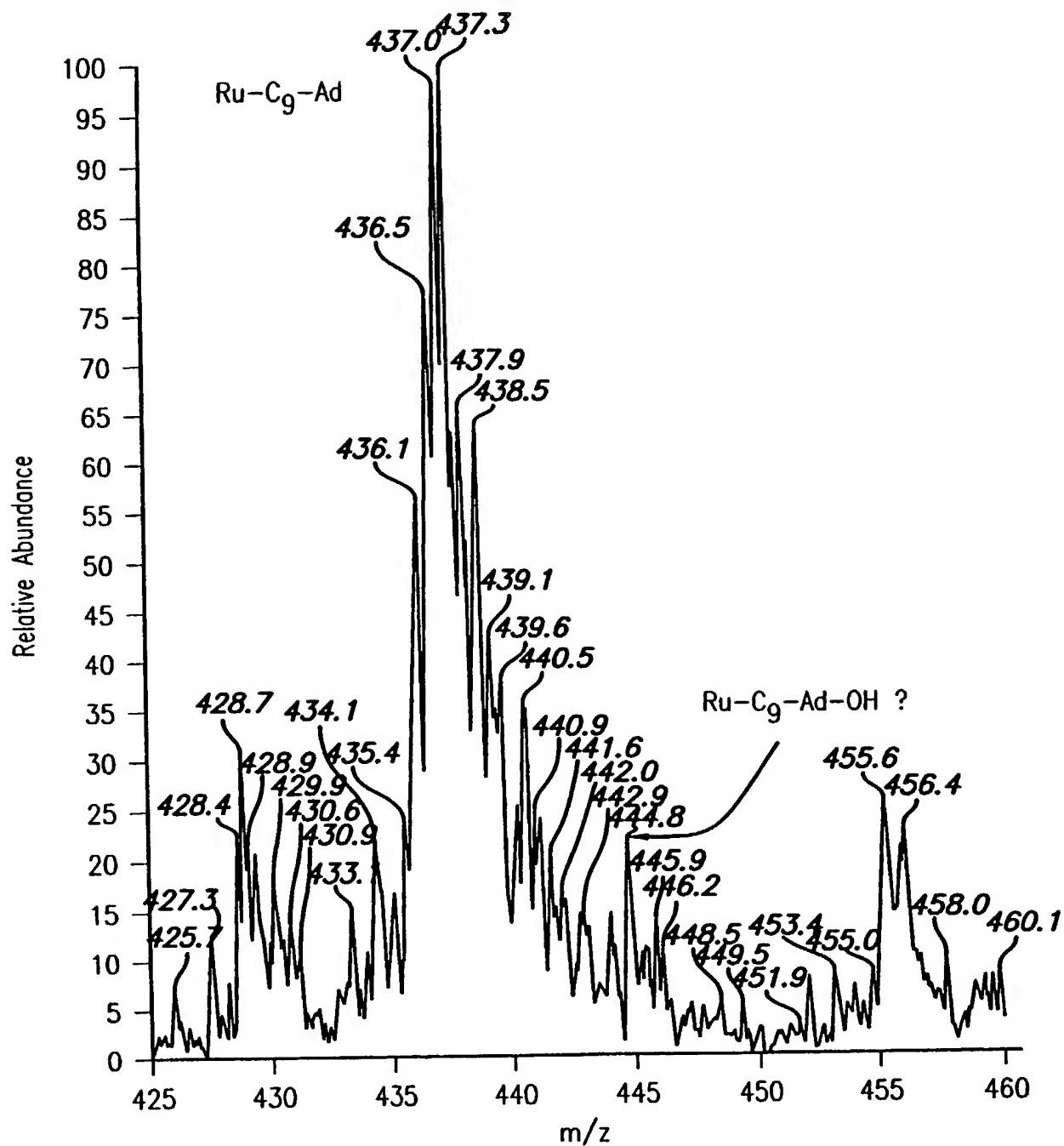
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FIG. 19



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FIG. 20



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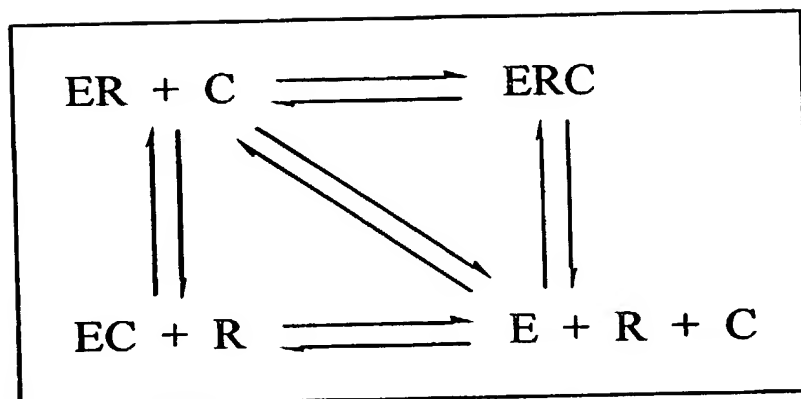


FIG. 21

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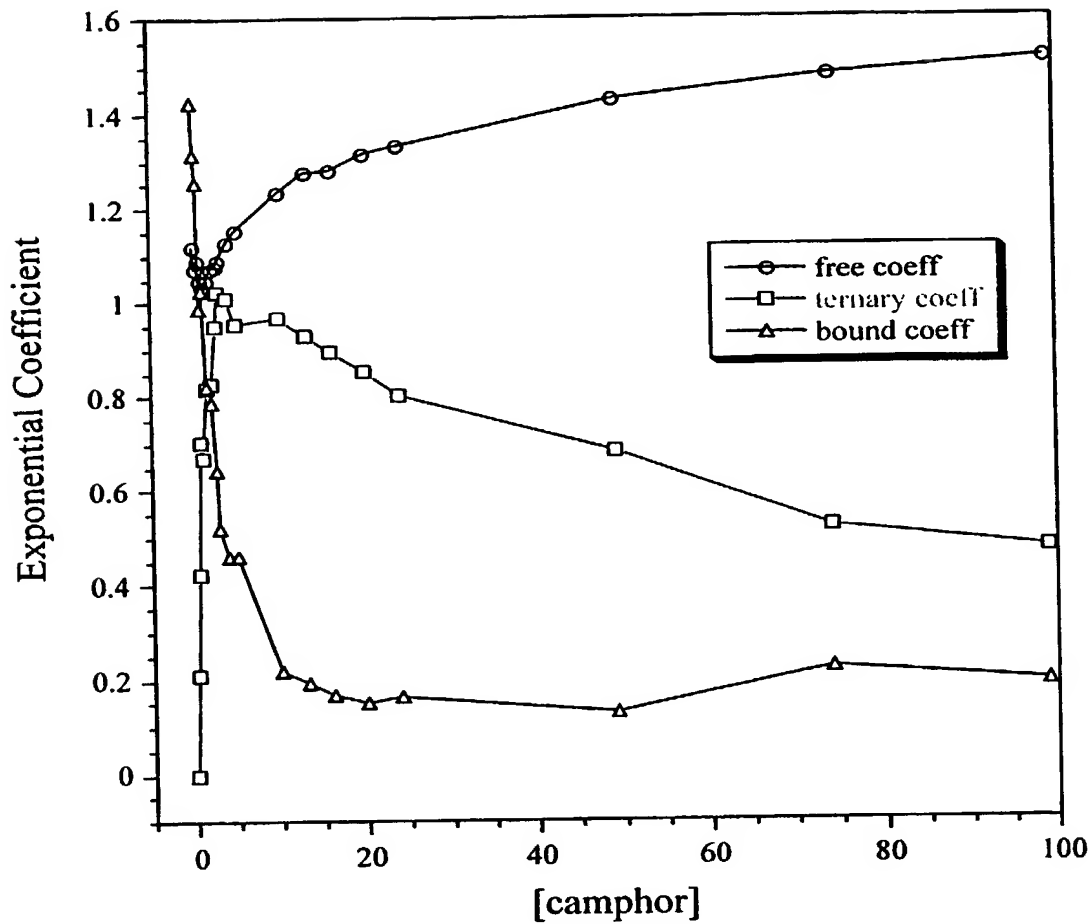


FIG. 22

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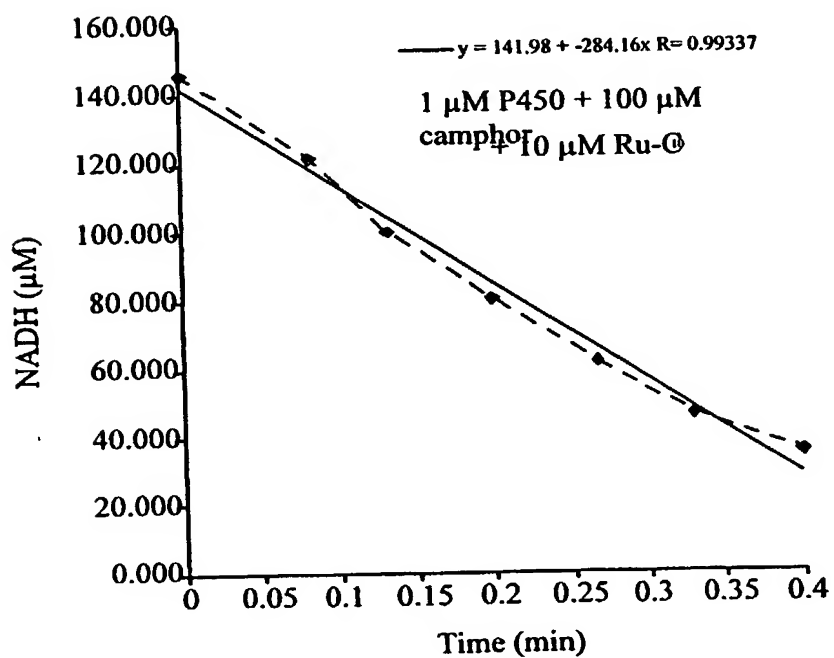
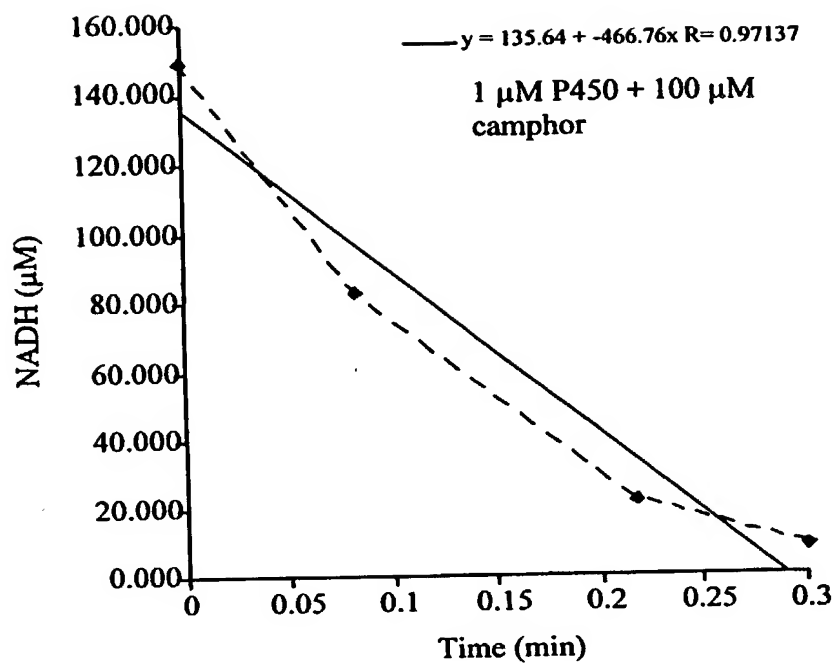


FIG. 22A

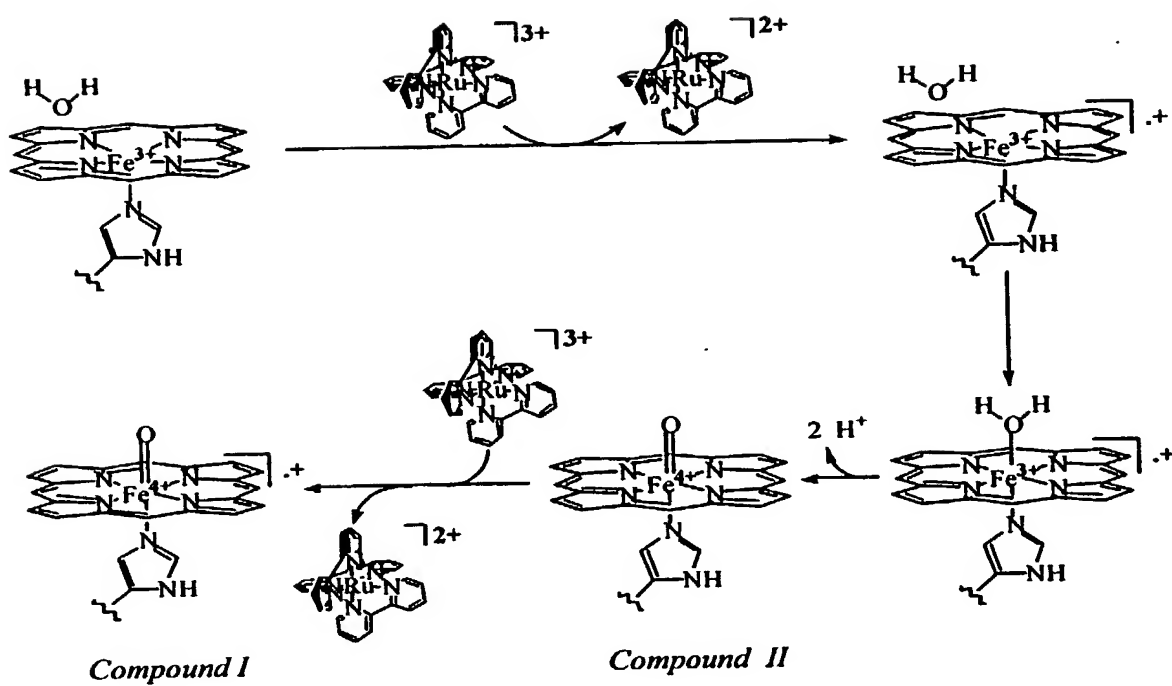
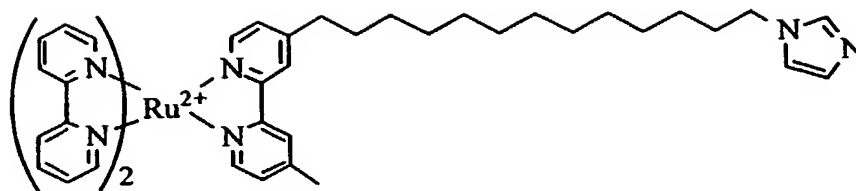


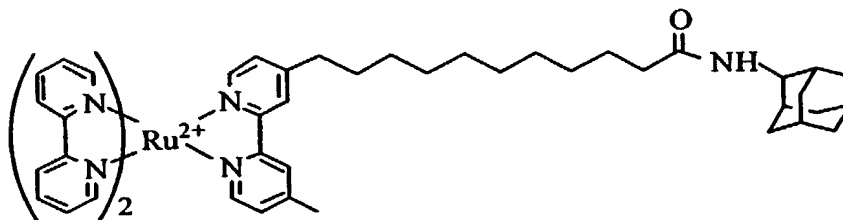
FIG. 23

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Ru-Im



Ru-Ad



Ru-EB

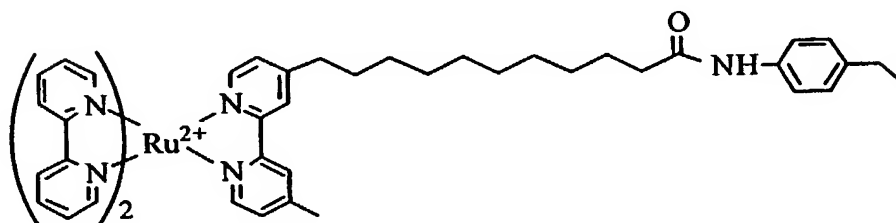


FIG. 24

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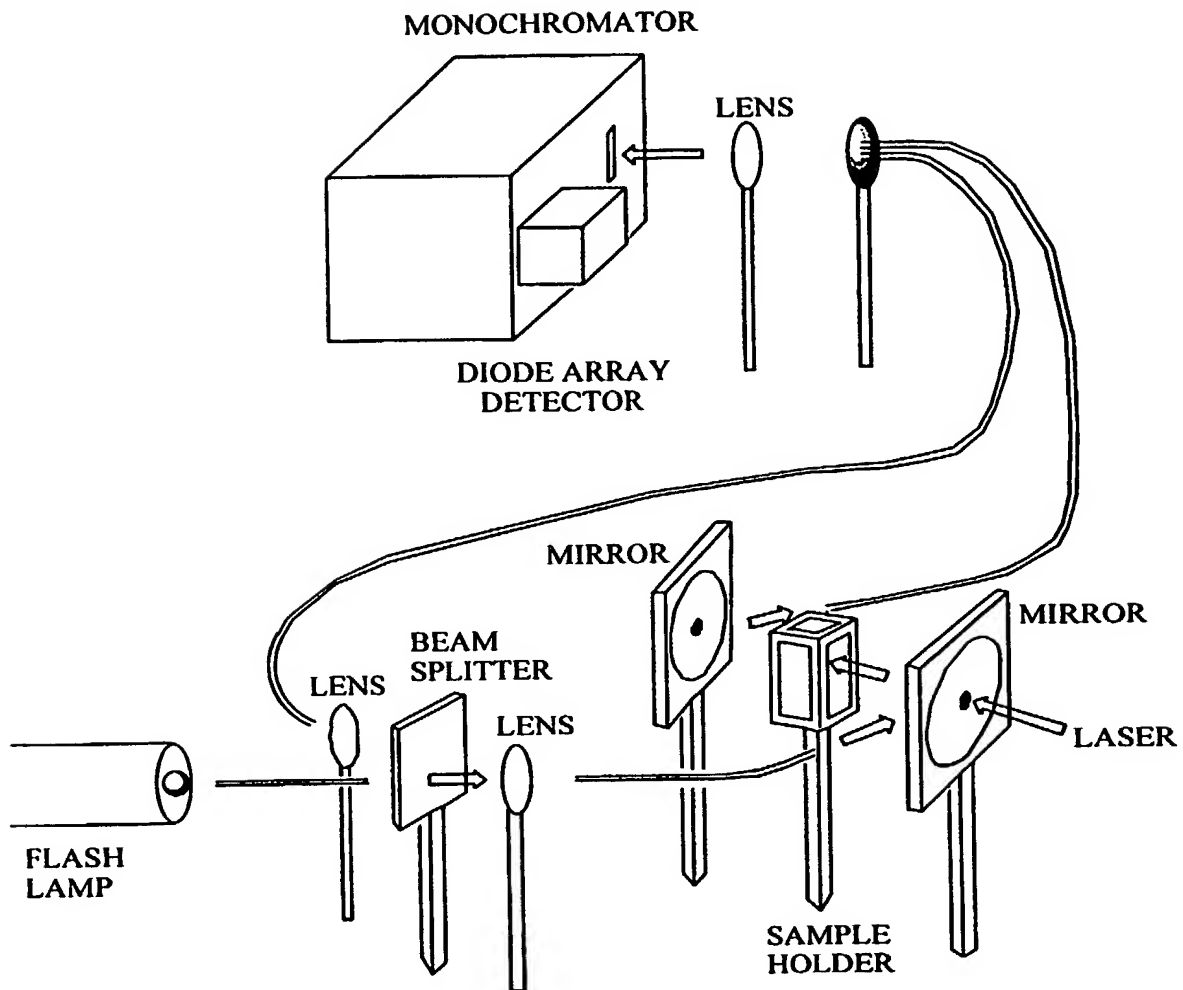


FIG. 25

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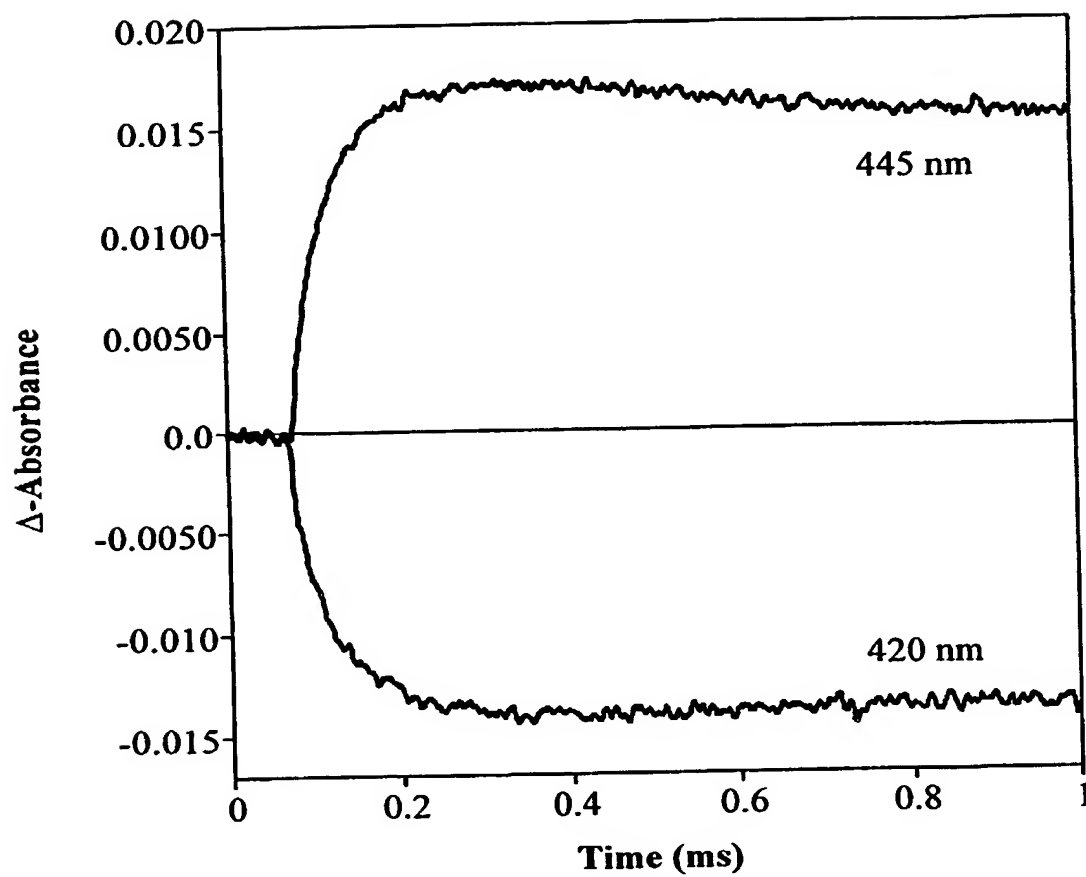


FIG. 26

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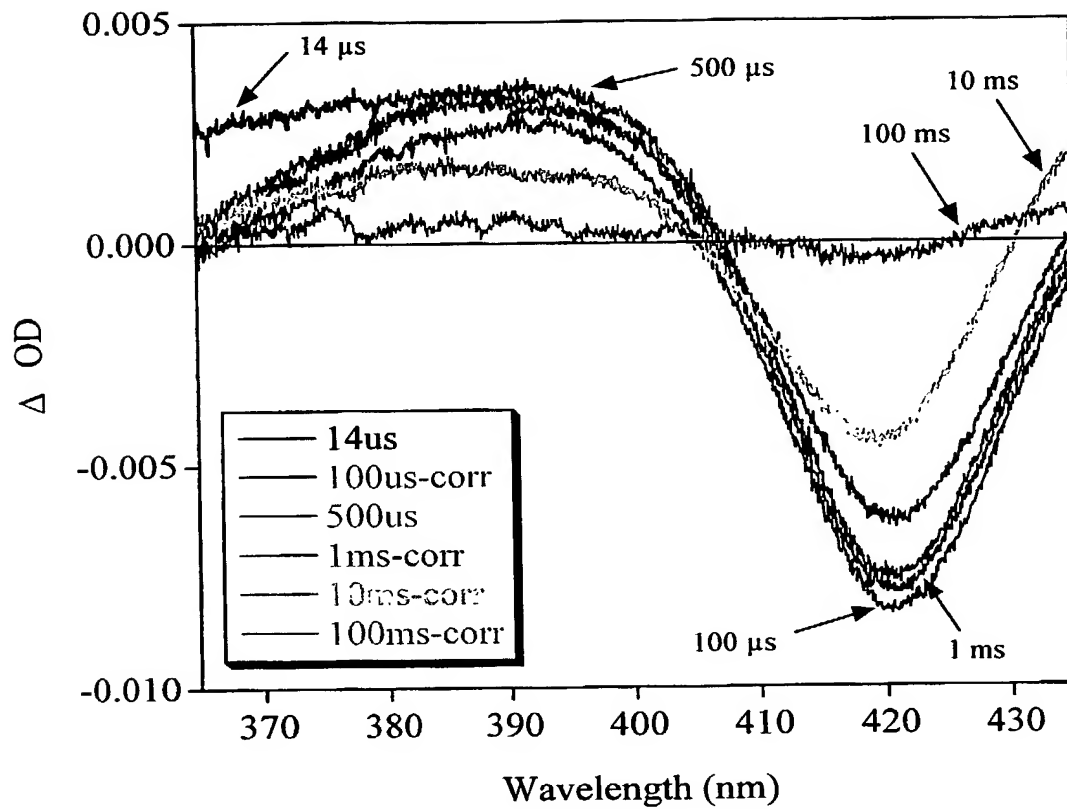


FIG. 27

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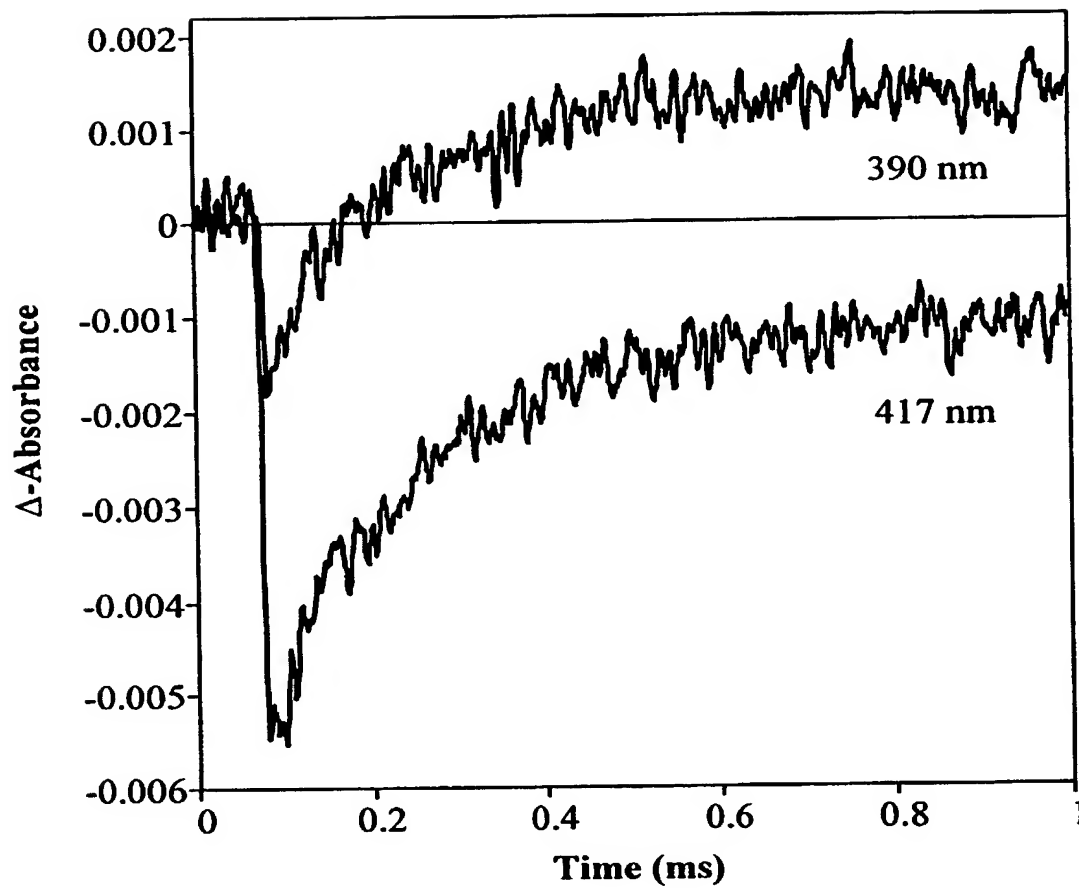


FIG. 28

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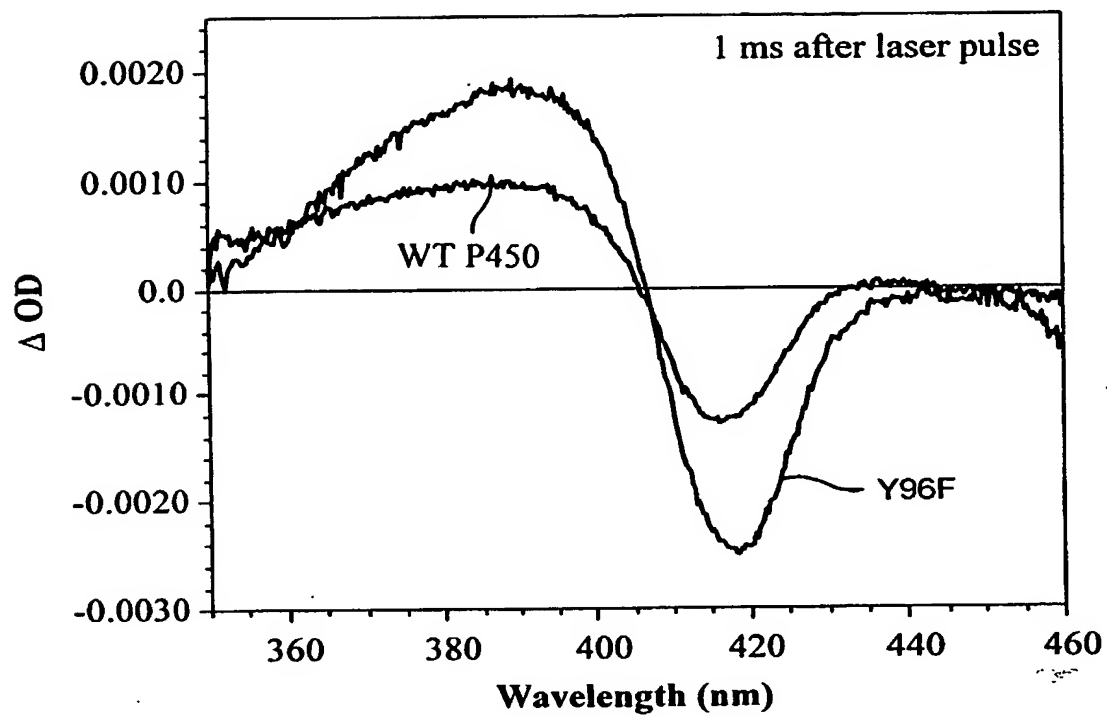


FIG. 29

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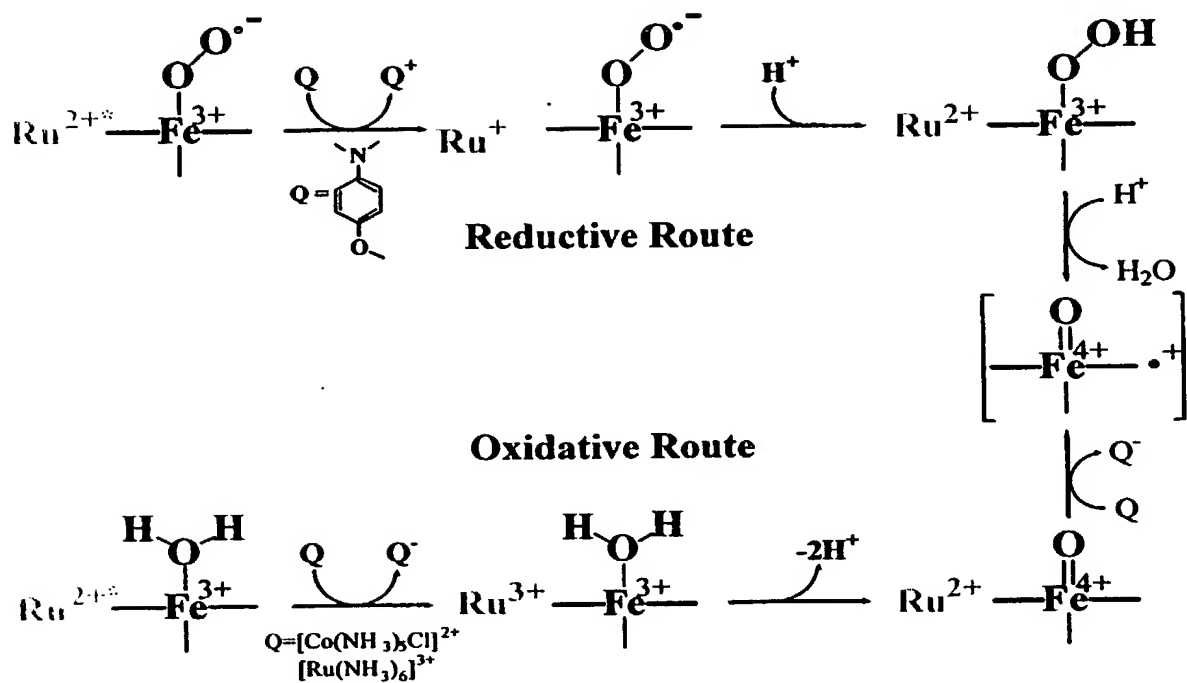


FIG. 30

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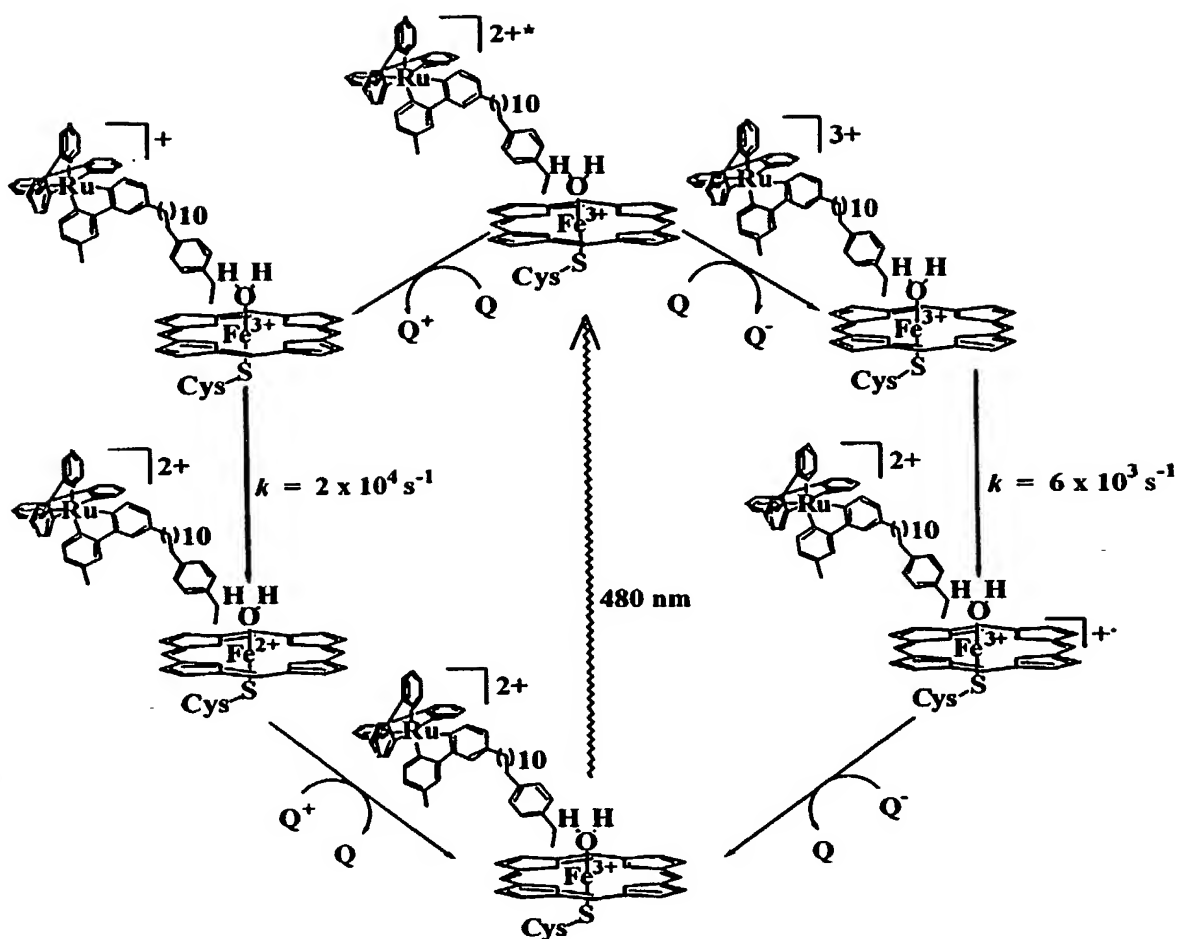


FIG. 31

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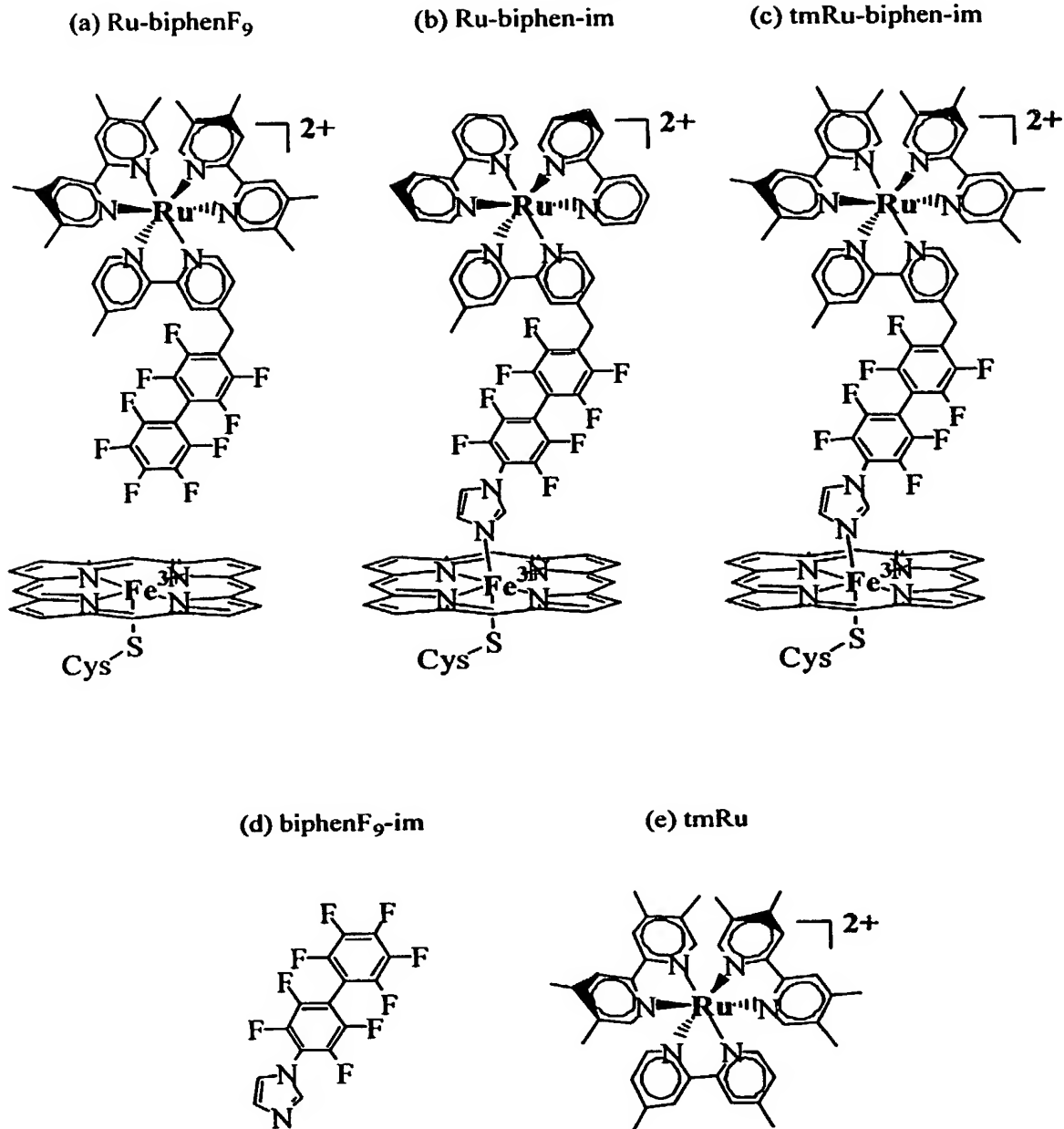


FIG. 32

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Ru	$K_D(\mu\text{M})$	$k_d(\text{s}^{-1})^a$	$k_{en}(\text{s}^{-1})^b$	$k_{ET}(\text{s}^{-1})^c$	$R_0(\text{\AA})$	Ru-Fe(\AA) ^d
(a)	0.9	4.6 E6	8.0 E6	N/A	18.8	19.7
(b)	3.7 (0.4)	2.0 (0.1) E6	8.5 E6	1 E6	22.1	17.7
(c)	0.5 (0.1)	4.6 (0.1) E6	9.5 E6	8 E6	18.0	17.7

*Uncertainties are given in parentheses, and constitute the standard deviation of four different measurements.

^aIntrinsic rate of excited-state decay of the Ru complex (in the absence of P450).

^bRate of energy transfer in the presence of P450, calculated from the emission profile. For (b) and (c) assumptions were made based on (a), k_{obs} , and reasonable Ru-Fe distances.

^cCalculated rate of electron transfer in the presence of P450, based on the observed yield of ET products. Actual yields (and rates), however, are probably much higher. Yields were calculated using extinction coefficients at 420 and 445 nm for Fe^{2+} while subtracting contributions from Ru^{3+} and Ru^{2+*} .

^dThe metal-metal (Ru-Fe) distance serves as a useful representation of the D-A pair.

FIG. 33

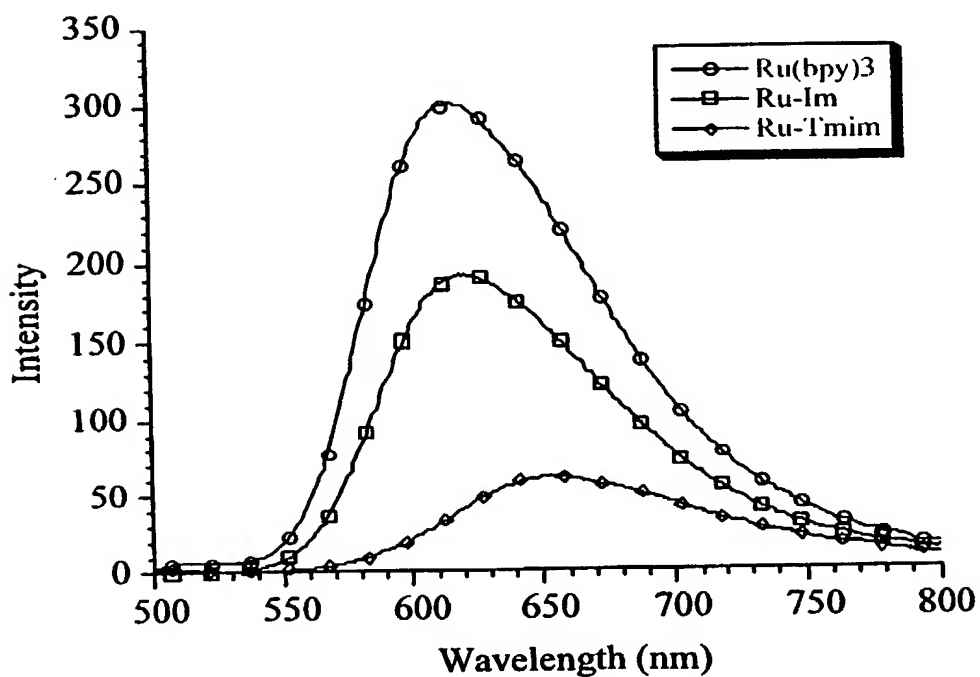
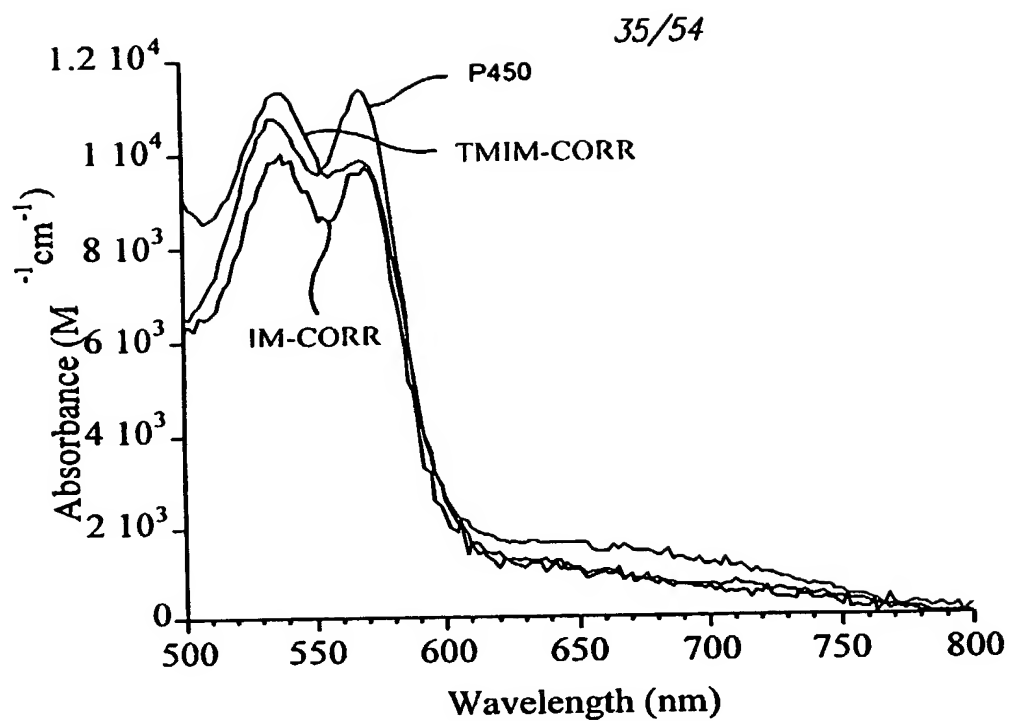


FIG. 34

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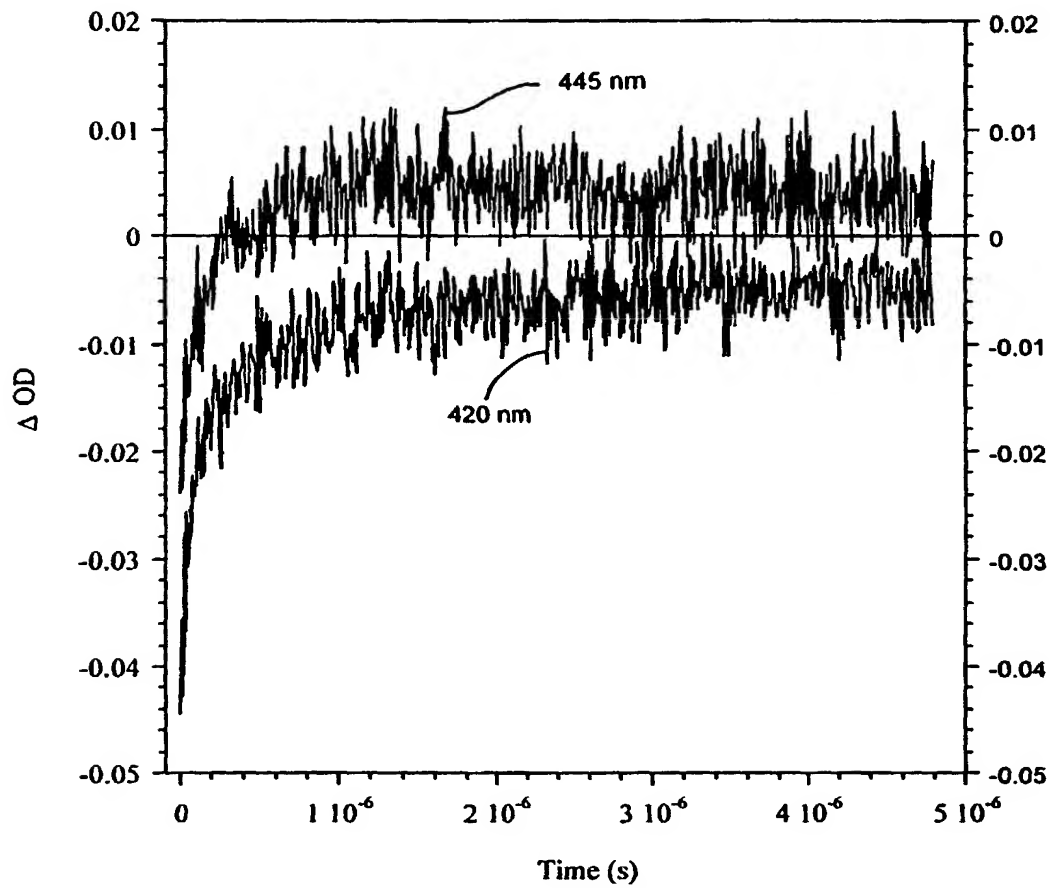


FIG. 35

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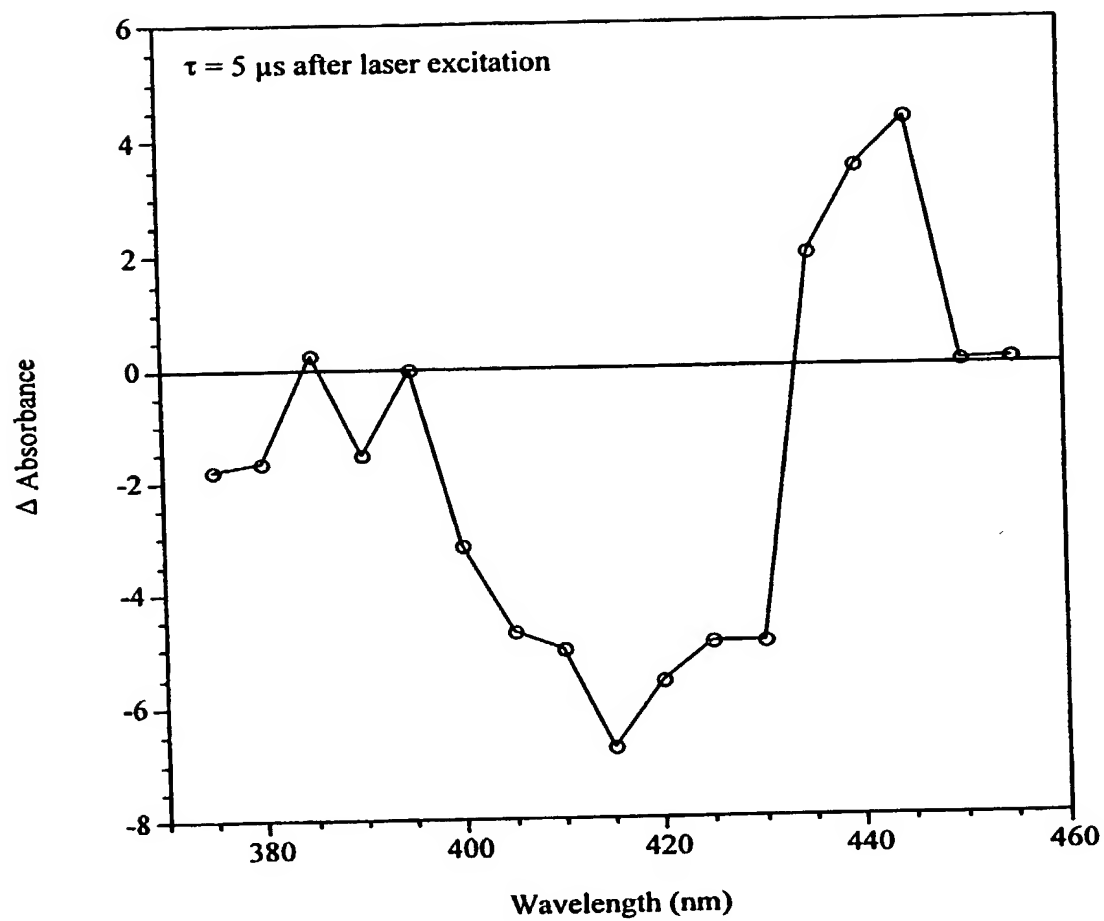


FIG. 36

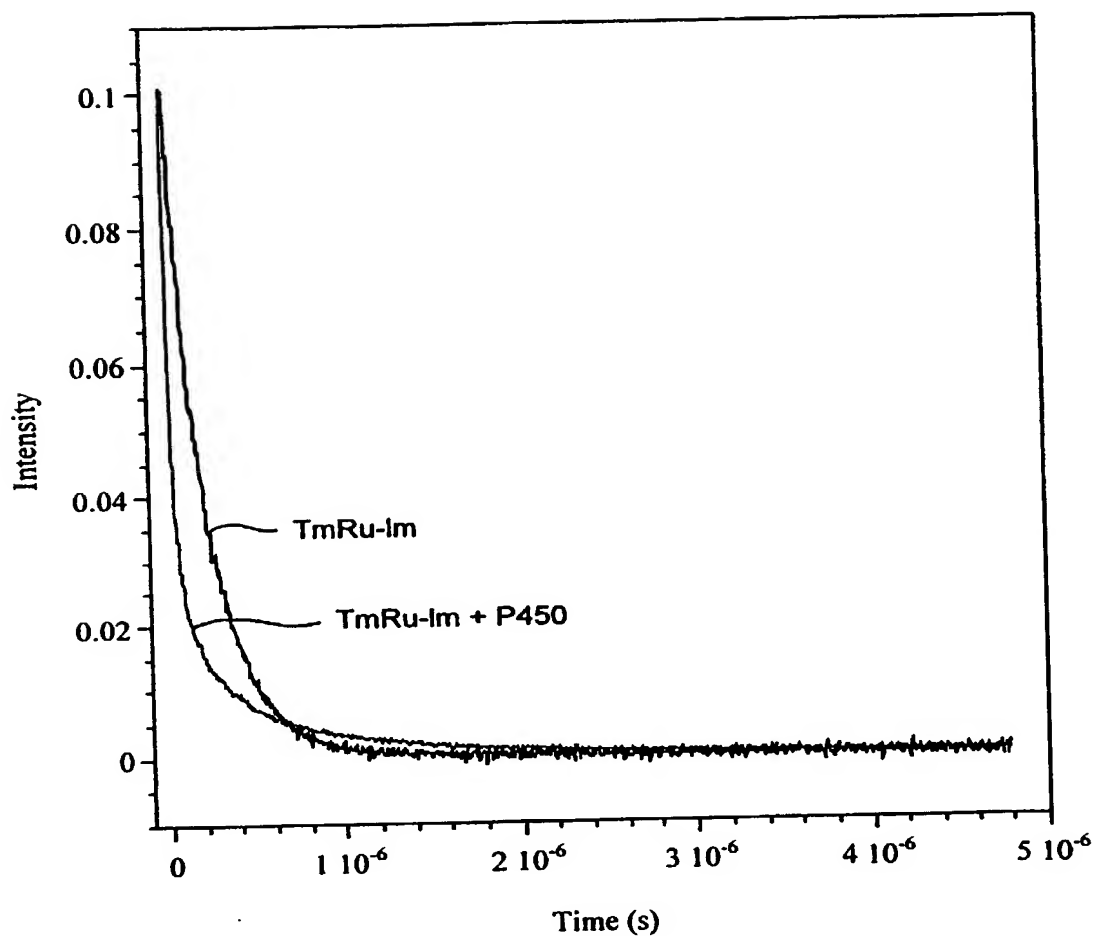


FIG. 37

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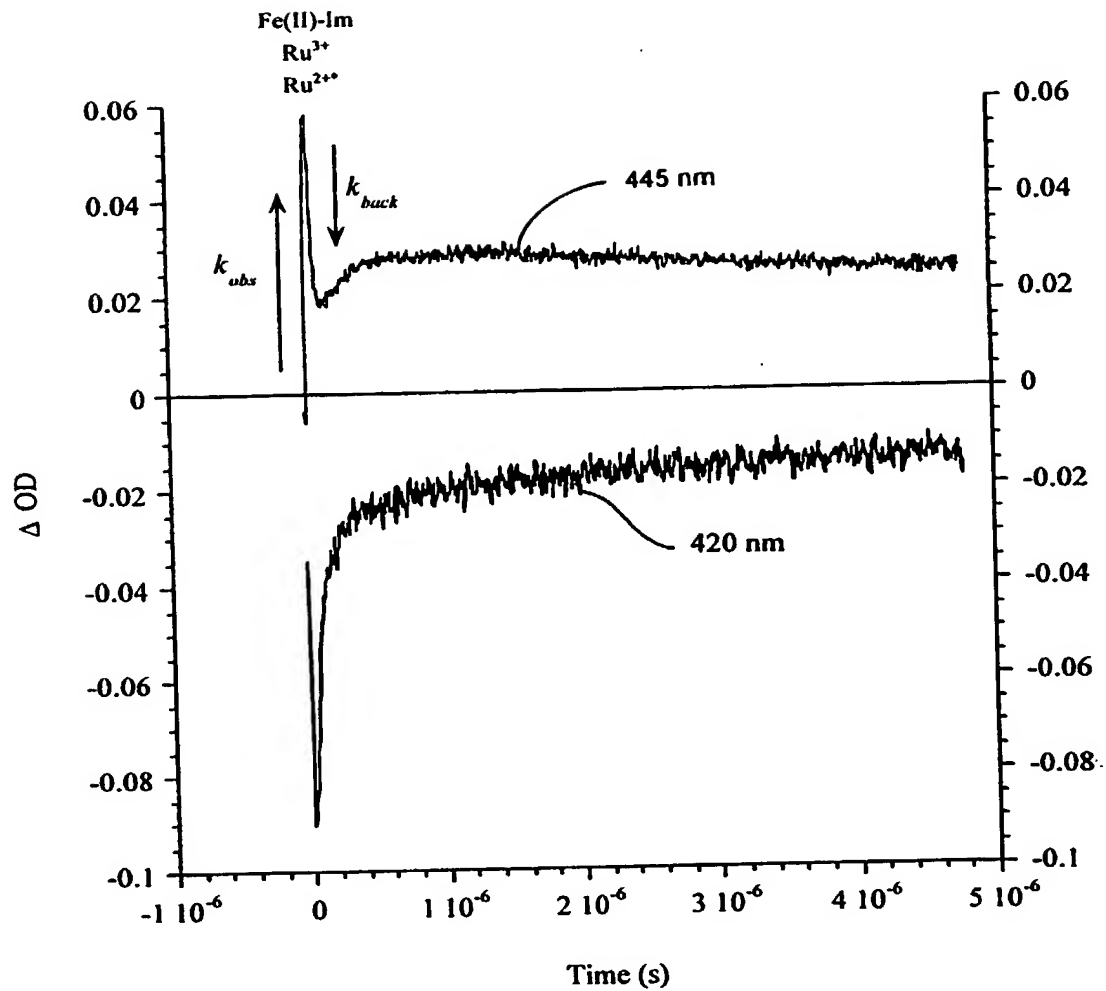


FIG. 38

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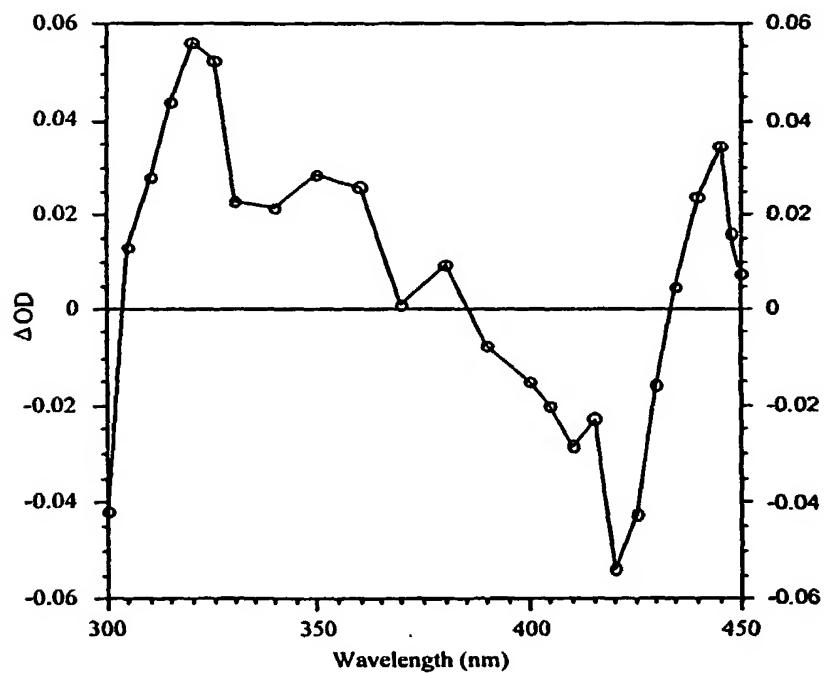
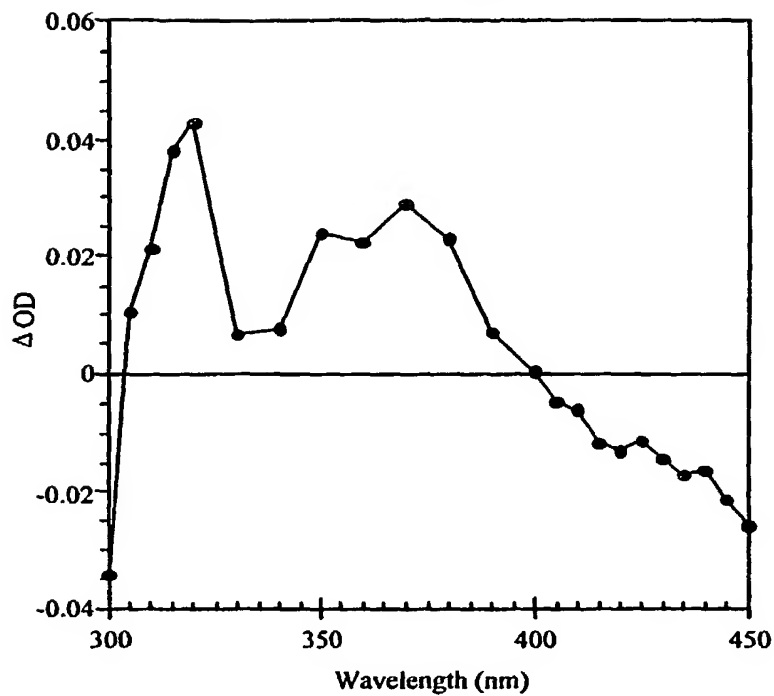


FIG. 39

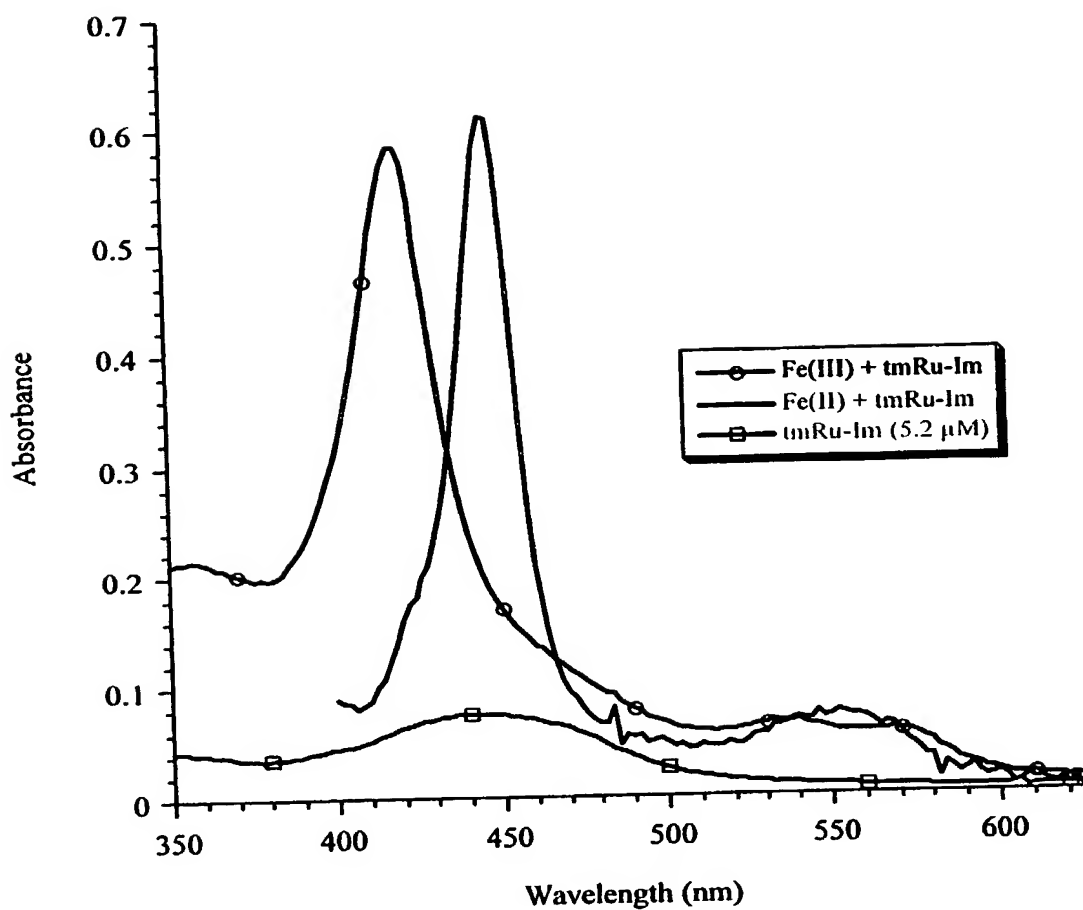


FIG. 40

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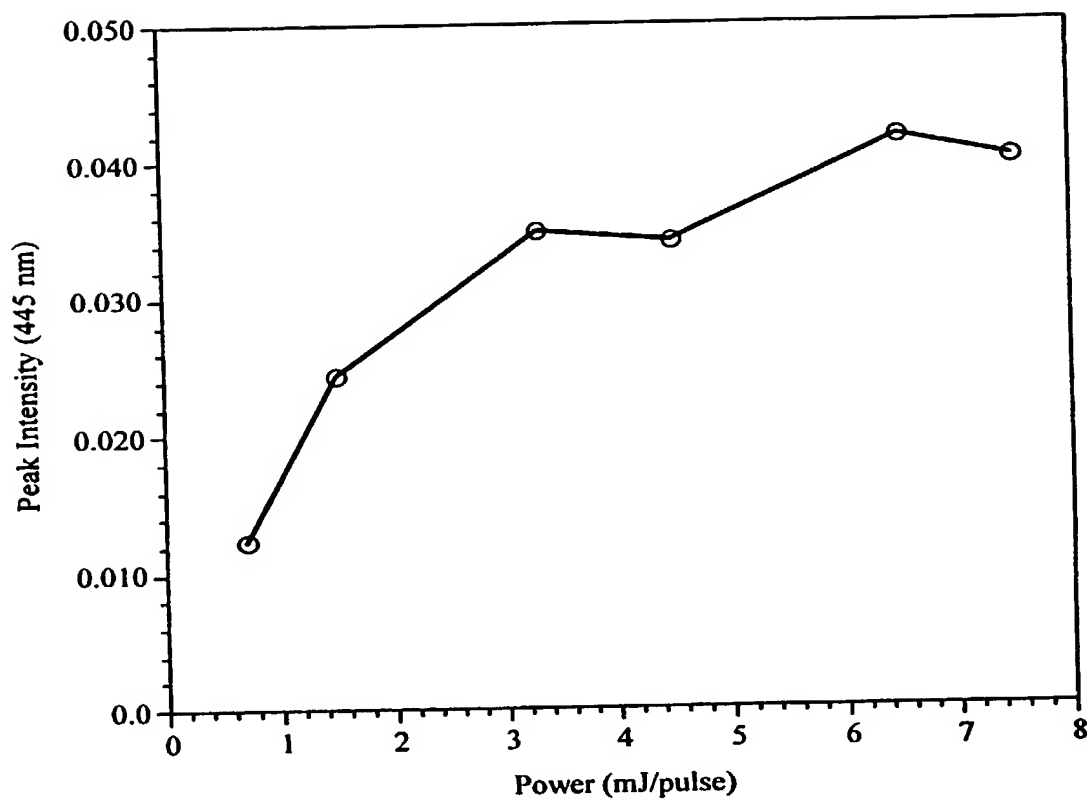


FIG. 41

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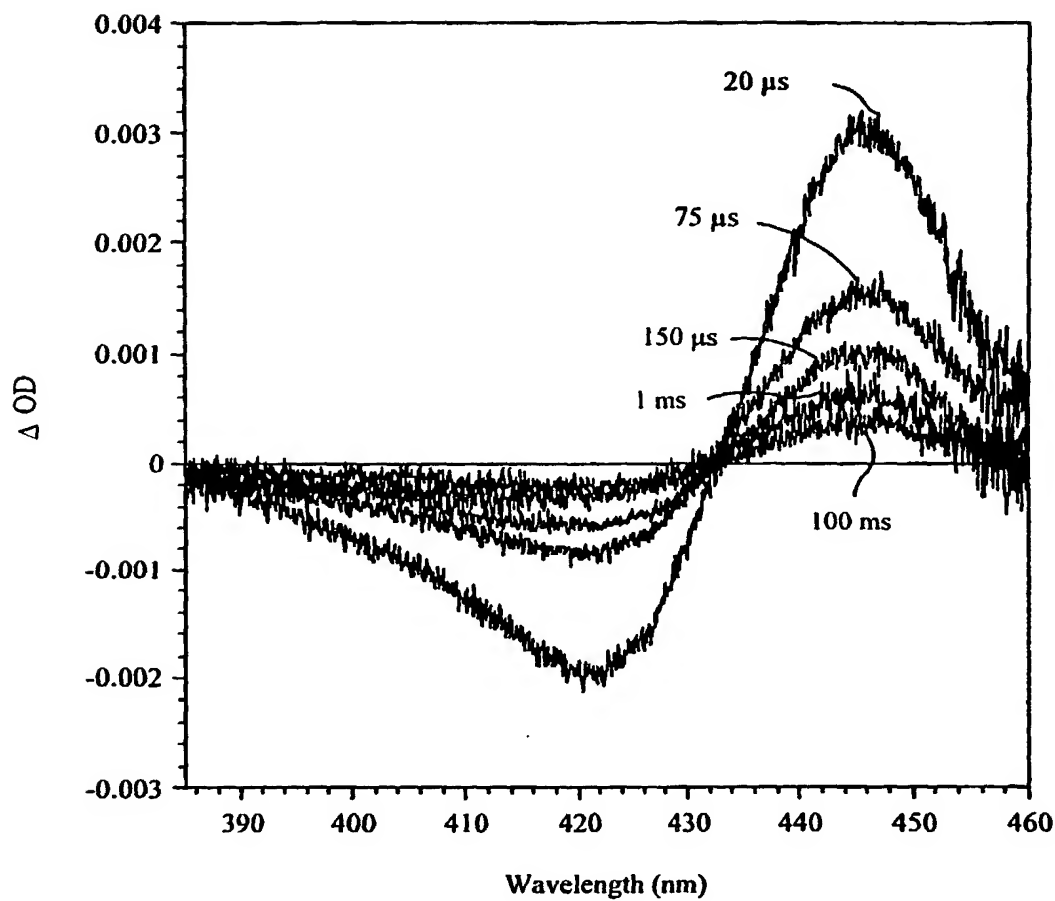


FIG. 42

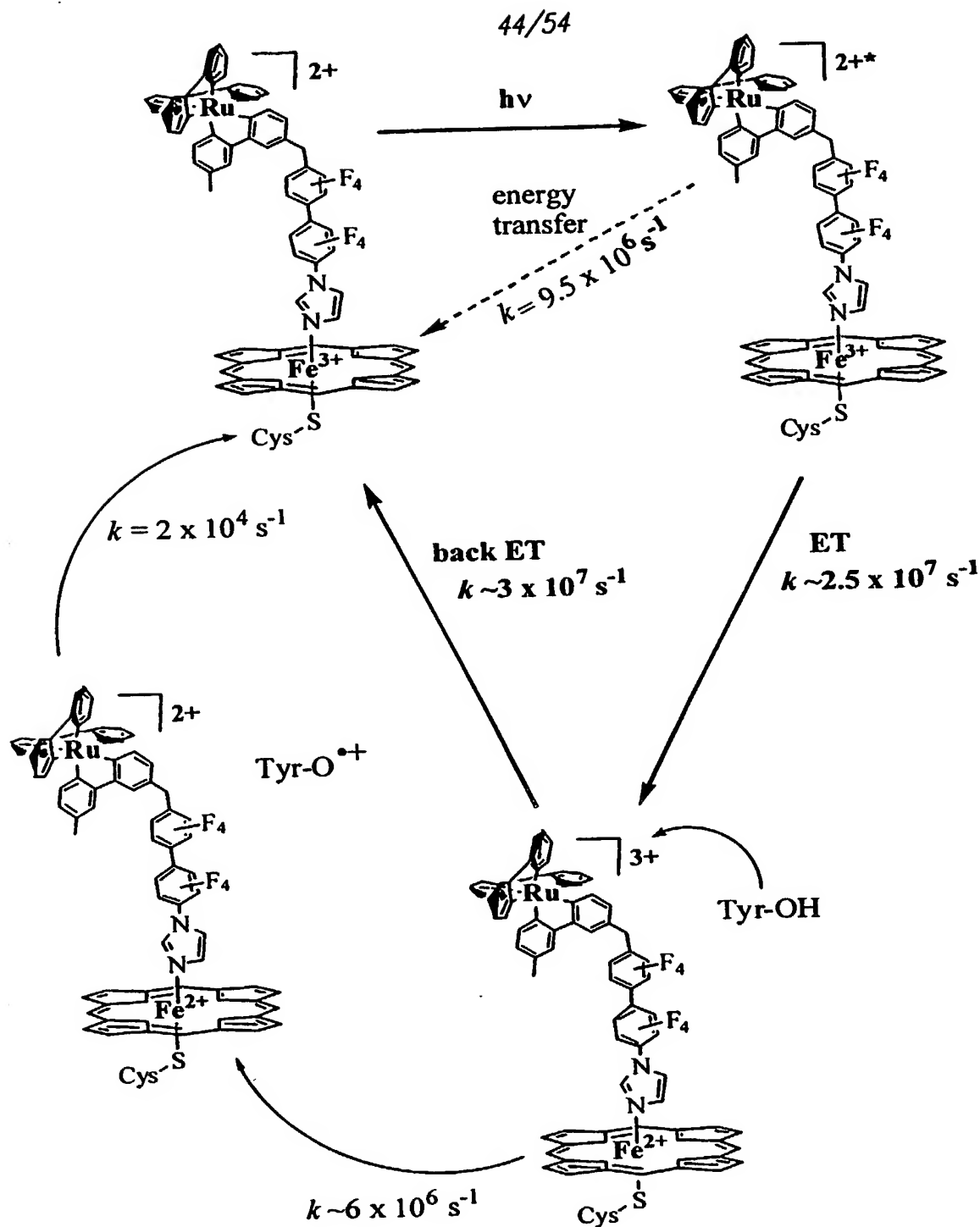


FIG. 43

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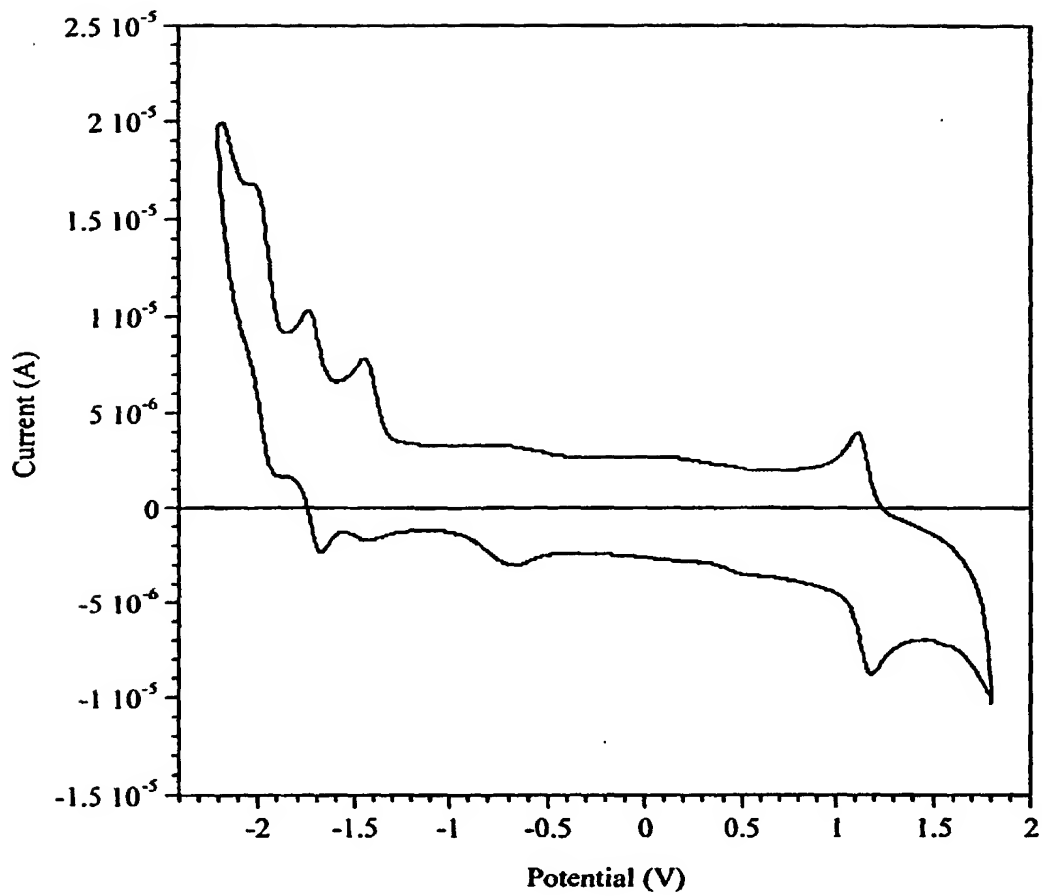


FIG. 44

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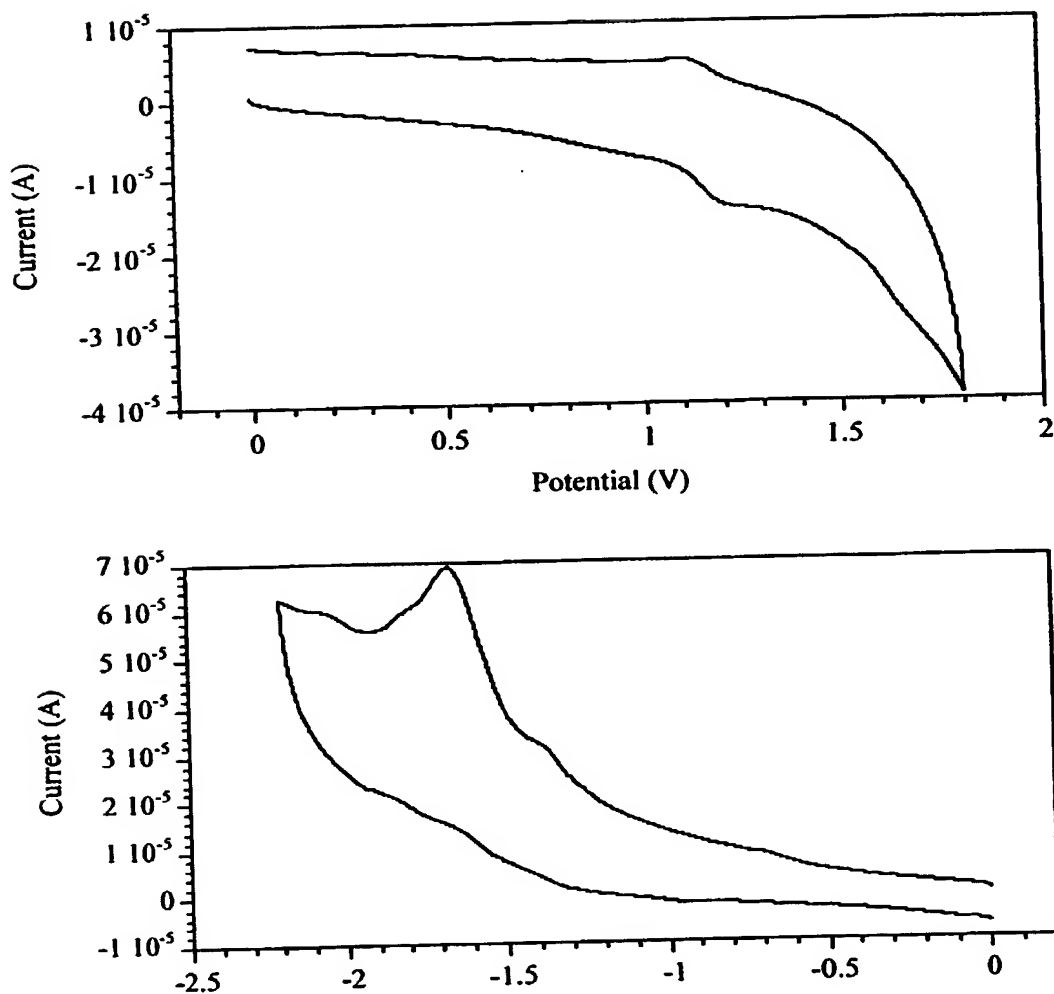


FIG. 45

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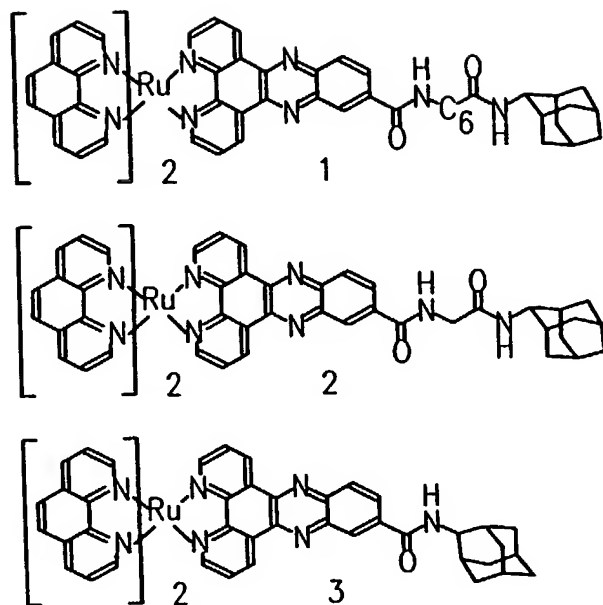


FIG. 46

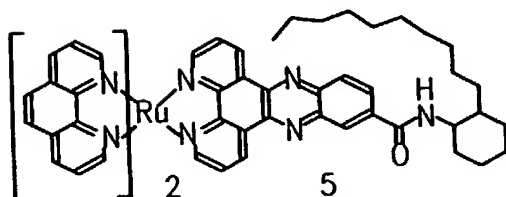


FIG. 50

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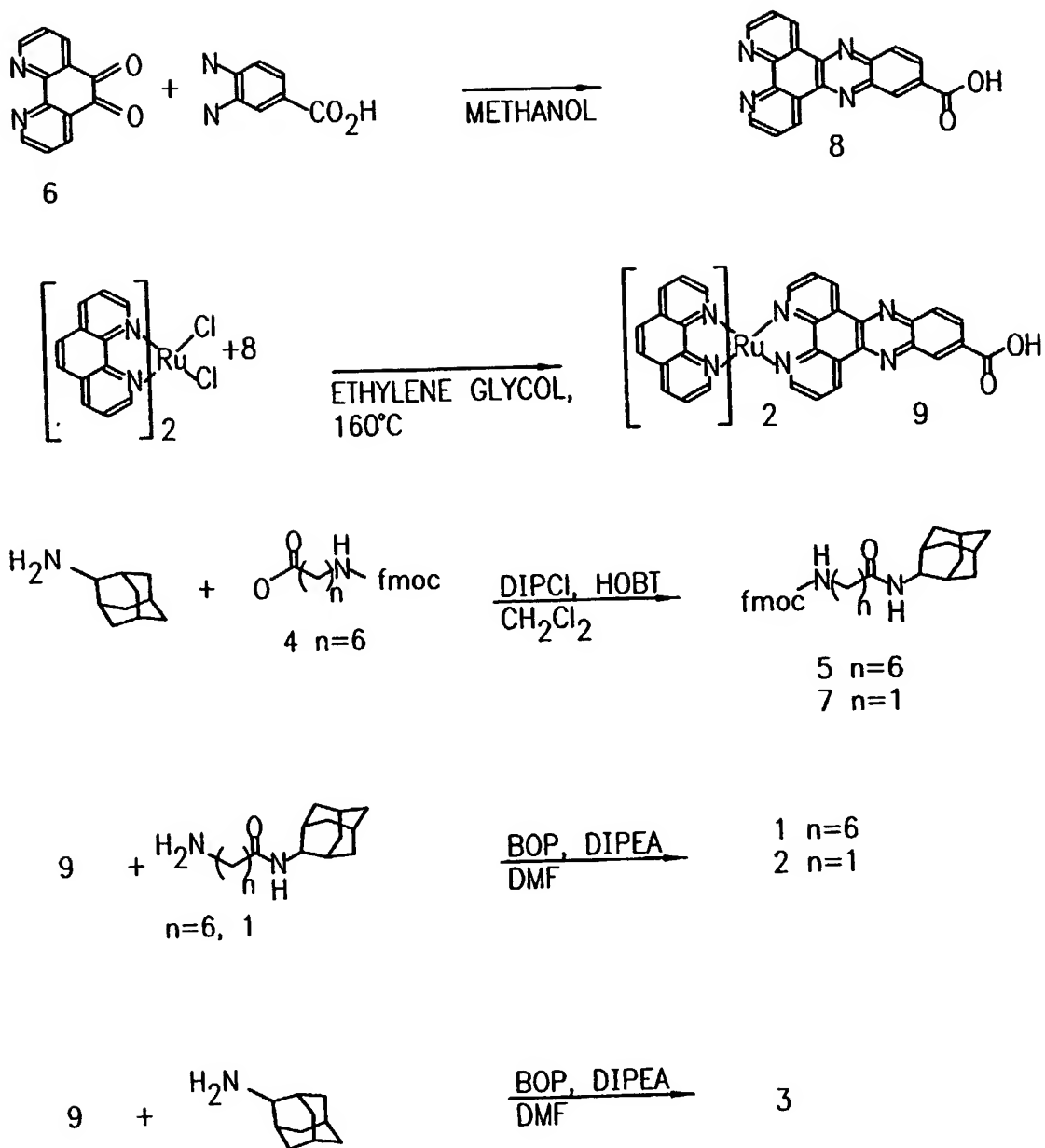


FIG. 47

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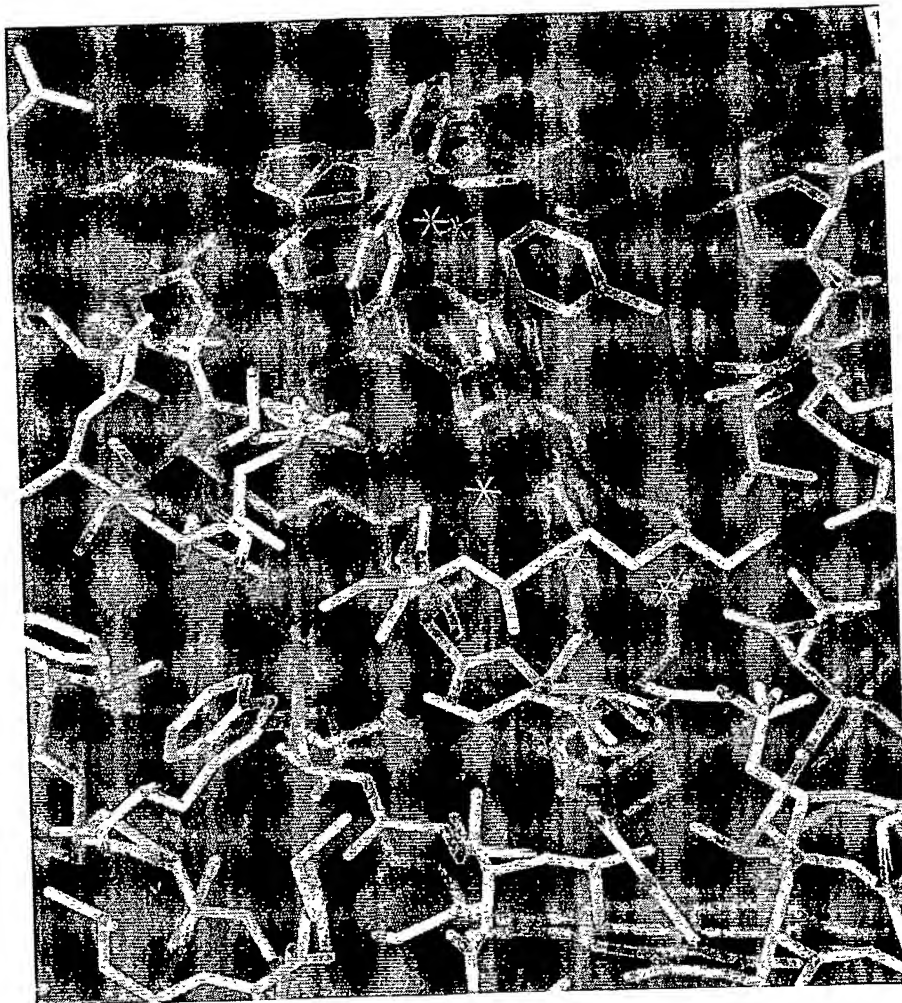


FIG. 48

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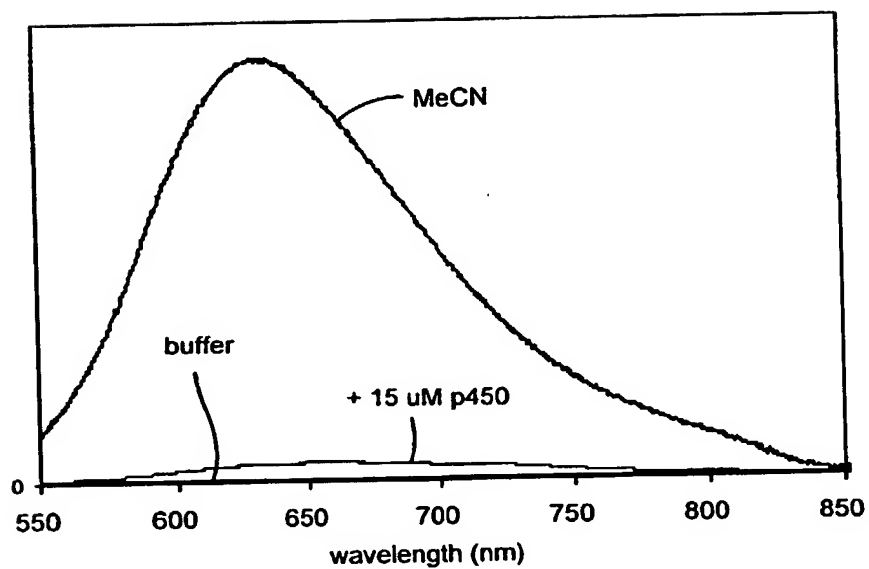


FIG. 49

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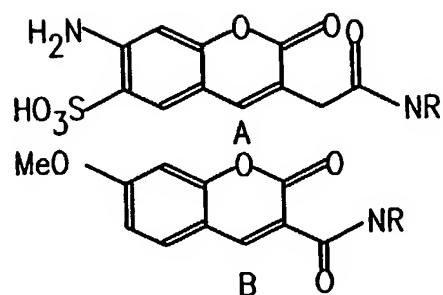


FIG. 51

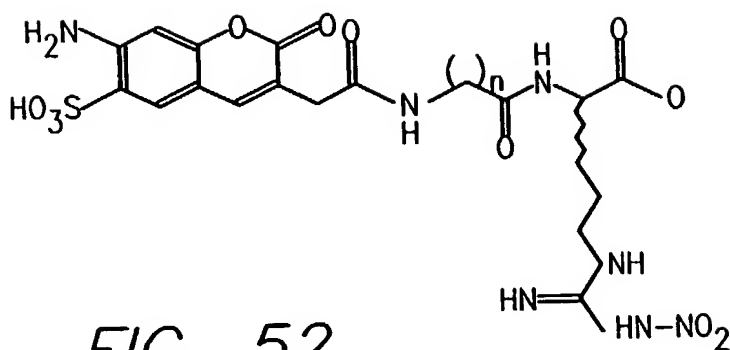


FIG. 52

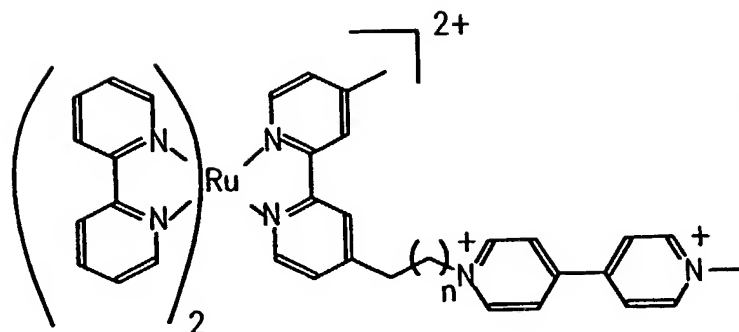
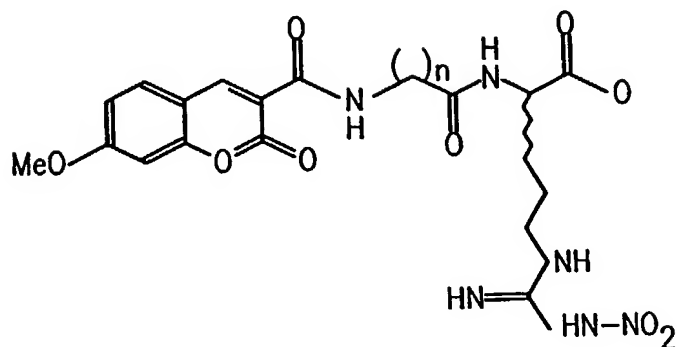


FIG. 55

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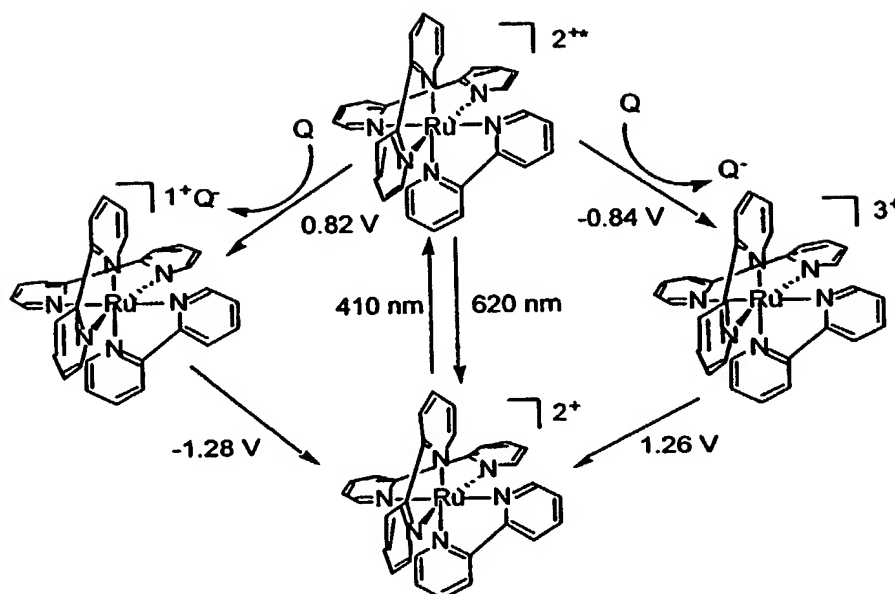
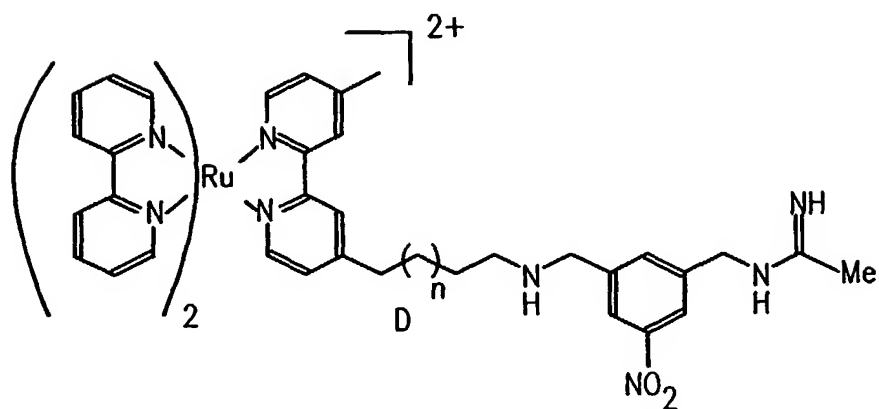
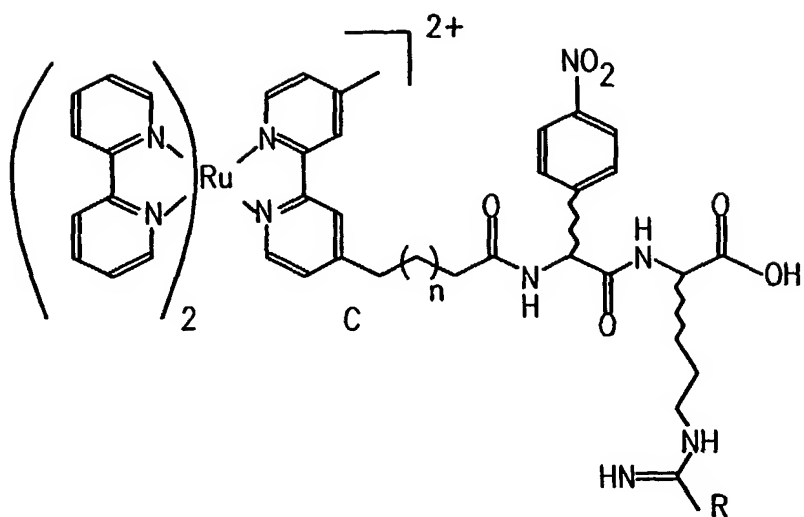


FIG. 53

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FIG. 54



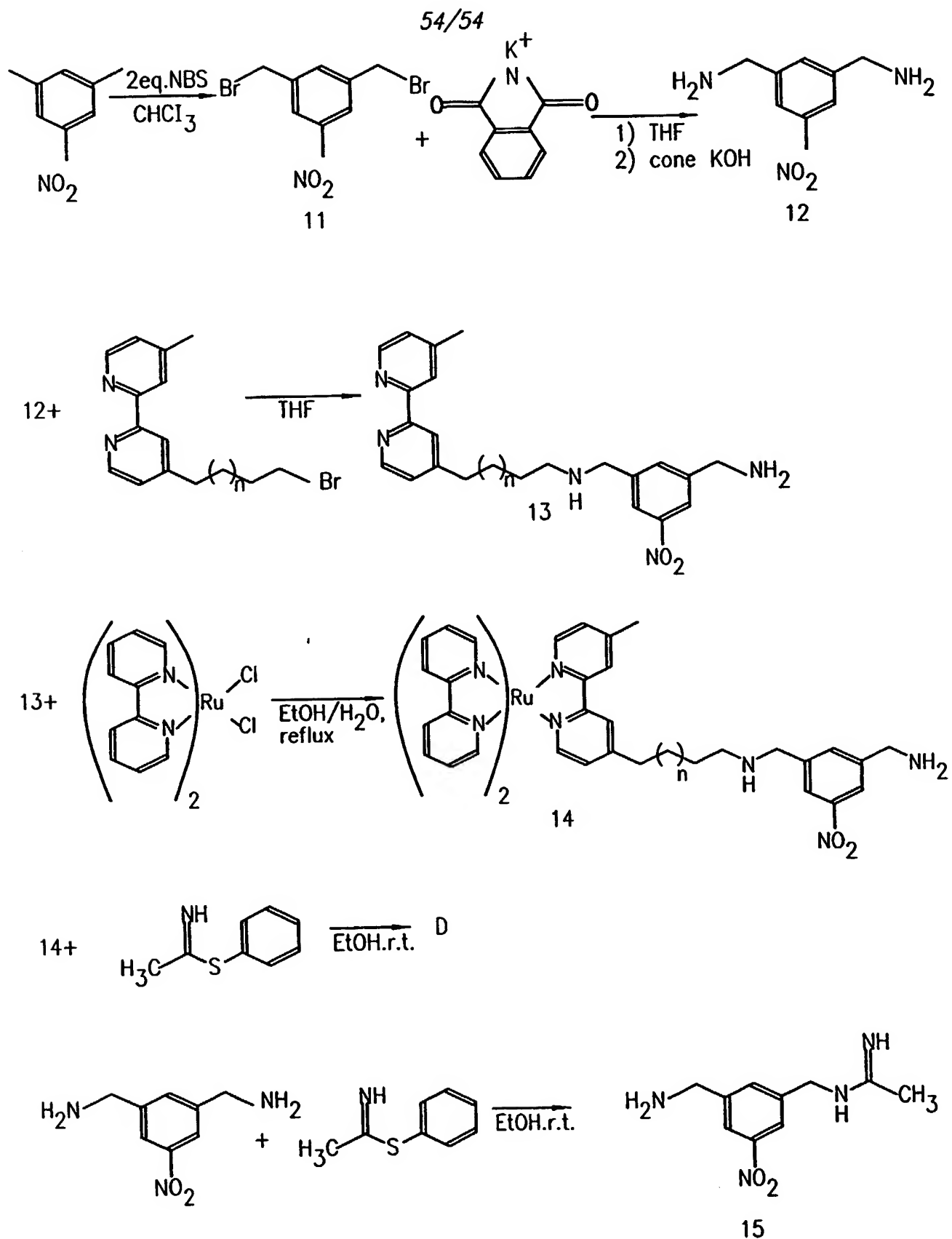


FIG. 56
SUBSTITUTE SHEET (RULE 26)

Attorney Docket No. CIT1490-3

DECLARATION FOR PATENT APPLICATION

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship is as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled DETECTION OF BIOMOLECULES BY SENSITIZER-LINKED SUBSTRATES, the specification of which

_____ is attached hereto.
X was filed on January 17, 2002 (Attorney Docket No. CIT1490-3)
as U.S. Application Serial No. 10/031,532
and was amended on _____
if applicable (the "Application").

I hereby authorize and request insertion of the application serial number of the Application when officially known.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

With respect to the Application, I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

<u>60/144,488</u> (Application Serial No.)	<u>July 19, 1999</u> (Filing Date)
<u>60/149,278</u> (Application Serial No.)	<u>August 16, 1999</u> (Filing Date)

Attorney Docket No. CIT1490-3

60/192,703 March 28, 2000
(Application Serial No.) (Filing Date)

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

COUNTRY	APPLICATION NO.	FILING DATE	PRIORITY CLAIMED
<u>US</u>	<u>PCT/US00/19821</u>	<u>July 19, 2000</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of first inventor: 10 Harry B. Gray

Inventor's signature: [Signature]

Date: 20 April 2002

Residence: 1415 East California Boulevard, Pasadena, California 91106 USA CA

Citizenship: USA

Post Office Address: 1415 East California Boulevard, Pasadena, California 91106 USA

Full name of second inventor: 2 Brian R. Crane

Inventor's signature: _____

Date: _____

Residence: 719 N. Aurora Street, Ithaca, New York USA NY

Citizenship: USA

Attorney Docket No. CIT1490-3

60/192,703 March 28, 2000
 (Application Serial No.) (Filing Date)

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

COUNTRY	APPLICATION NO.	FILING DATE	PRIORITY CLAIMED
<u>US</u>	<u>PCT/US00/19821</u>	<u>July 19, 2000</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of first inventor: Harry B. Gray

Inventor's signature: _____

Date: _____

Residence: 1415 East California Boulevard, Pasadena, California 91106 USA

Citizenship: USA

Post Office Address: 1415 East California Boulevard, Pasadena, California 91106 USA

Full name of second inventor: Brian R. Crane

Inventor's signature: 

Date: 15/04/02

Residence: B.C. 105 McIntyre PL
719 N. Aurora Street, Ithaca, New York USA

Citizenship: USA

Attorney Docket No. CIT1490-3

Post Office Address: 719 N. Aurora Street, Ithaca, New York USA

Full name of third inventor: 3-D Jay R. Winkler

Inventor's signature: J.R. Winkler

Date: 25 April, 2002

Residence: 1110 N. Chester Avenue, Pasadena, California 91104

Citizenship: USA

Post Office Address: 1110 N. Chester Avenue, Pasadena, California 91104

Full name of fourth inventor: 4-D Ivan Julian Dmochowski

Inventor's signature: _____

Date: _____

Residence: 97 N. Holliston Avenue, Pasadena, California 91106

Citizenship: USA

Post Office Address: 97 N. Holliston Avenue, Pasadena, California 91106

Full name of fifth inventor: 5-D Jonathan J. Wilker

Inventor's signature: _____

Date: _____

Residence: 2421 Kestral Blvd., West Lafayette, Indiana 47906

Citizenship: USA

Post Office Address: 2421 Kestral Blvd., West Lafayette, Indiana 47906

Full name of sixth inventor: Alexander Robert Dunn

Inventor's signature: _____

Date: _____

Post Office Address: 719 N. Aurora Street, Ithaca, New York USA

Full name of third inventor: Jay R. Winkler

Inventor's signature: _____

Date: _____

Residence: 1110 N. Chester Avenue, Pasadena, California 91104

Citizenship: USA

Post Office Address: 1110 N. Chester Avenue, Pasadena, California 91104

Full name of fourth inventor: Ivan Julian Dmochowski

Inventor's signature: IJ Dmochowski

Date: 2/5/02

Residence: 6641 Aldama St., Los Angeles, CA 90042
~~97 N. Holliston Avenue, Pasadena, California 91106~~

Citizenship: USA

Post Office Address: 6641 Aldama St., Los Angeles, CA 90042
~~97 N. Holliston Avenue, Pasadena, California 91106~~

Full name of fifth inventor: Jonathan J. Wilker

Inventor's signature: _____

Date: _____

Residence: 2421 Kestral Blvd., West Lafayette, Indiana 47906

Citizenship: USA

Post Office Address: 2421 Kestral Blvd., West Lafayette, Indiana 47906

Full name of sixth inventor: Alexander Robert Dunn

Inventor's signature: _____

Date: _____

Attorney Docket No. CIT1490-3

Post Office Address: 719 N. Aurora Street, Ithaca, New York USA

Full name of third inventor: Jay R. Winkler

Inventor's signature: _____

Date: _____

Residence: 1110 N. Chester Avenue, Pasadena, California 91104

Citizenship: USA

Post Office Address: 1110 N. Chester Avenue, Pasadena, California 91104

Full name of fourth inventor: Ivan Julian Dmochowski

Inventor's signature: _____

Date: _____

Residence: 97 N. Holliston Avenue, Pasadena, California 91106

Citizenship: USA

Post Office Address: 97 N. Holliston Avenue, Pasadena, California 91106

Full name of fifth inventor: Jonathan J. Wilker

Inventor's signature: 

Date: 4/17/02

Residence: 2421 Kestral Blvd., West Lafayette, Indiana 47906

Citizenship: USA

Post Office Address: 2421 Kestral Blvd., West Lafayette, Indiana 47906

Full name of sixth inventor: Alexander Robert Dunn

Inventor's signature: _____

Date: _____

ALL INFORMATION CONTAINED HEREIN IS UNCLASSIFIED

Attorney Docket No. CIT1490-3

Residence: 4495 Terrell Lane, Colorado Springs, Colorado 80908

Citizenship: USA

Post Office Address: 4495 Terrell Lane, Colorado Springs, Colorado 80908

